THE DIRECT EFFECTS OF DESOXYCORTICOSTERONE ON SKELETAL MUSCLE ELECTROLYTE METABOLISM

by

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I. GENERAL INTRODUCTION

The vital function of the adrenal glands has been recognized for over one hundred years. In 1855, Addison (cited by Thorn, 1942) described a clinical syndrome which resulted from adrenal gland destruction by tuberculosis. The following year, Brown-Séquard (cited by Thorn, 1942) demonstrated that the removal of both adrenals was promptly followed by death of the experimental animal.

The importance of the adrenal cortex and the relative unimportance of the adrenal medulla in maintaining life was indicated by several different approaches. Wheeler and Vincent (1912, cited by Houssay and Lewis, 1923) completely removed the left adrenal of a dog and excised one-half of the right adrenal. After cauterizing the medulla, the animal remained in good condition and had a longer life span than bilaterally adrenalectomized controls. Houssay and Lewis (1923) scooped out the adrenal medullas of dogs and found that the animals remained in good health. Histological examinations proved that all the medulla had been destroyed; however, chromaffin tissue found outside the adrenal medulla was not considered as a possible source of life-maintaining hormone. Wislocki and Crowe (1924), recognizing that non-adrenal chromaffin tissue might be important, removed surgically all the abdominal chromaffin tissue in addition to destroying the adrenal medulla with radium. They found that as long as one-fifth of one adrenal cortex was unharmed the animal remained in good condition.

Other evidence mustered to show that the medulla is relatively unimportant in maintaining life includes the following observations:

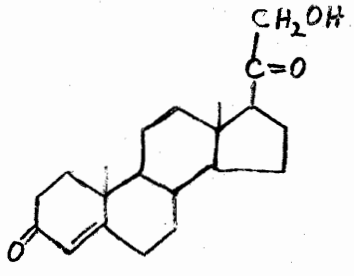
(1) epinephrine did not maintain life in a bilaterally adrenalectomized animal (Banting and Gairns, 1926; Hartman, 1926); (2) denervation of the medulla caused a disappearance of adrenaline in peripheral blood, but no effect was observed on the survival of dogs (Stewart and Rogoff, 1917); (3) extirpation of the inter-renal bodies of elasmobranches (which correspond to the adrenal cortex in mammals) caused death, even though chromaffin tissue still remained (Biedl, 1913; Kisch, 1928; both cited by Hartman and Brownell, 1949); and (4) destroying cortical cell integrity with a freezing ethyl chloride spray killed healthy rabbits (Marine and Baumann, 1921).

Once the importance of the adrenal cortex had been firmly established, many attempts to unravel the complexities of the physiology of this organ were initiated. An extract of hog adrenal glands was used by Osler (1896, cited by Thorn et al., 1953) for the treatment of Addison's Disease, even though he was not aware of the importance of the cortex for life. Hartman, MacArthur, and Hartman (1927) and Rogoff and Stewart (1927) independently reported the preparation of aqueous or glycerol extracts of adrenal cortex that prolonged the life of adrenalectomized cats and dogs respectively. Swingle and Pfiffner (1930) and Hartman and Bownell (1930) used organic solvents to secure a more potent cortical extract, and found more suitable procedures for removing contaminating epinephrine from extracts. Thus, higher doses of a more effective compound allowed very successful demonstrations of the benefits of these extracts in adrenal insufficiency (Rowntree and Greene, 1930; Hartman et al., 1931). Excessive adrenocortical activity caused by adrenal cortical tumors or hypophyseal neoplasms also yielded information about the physiological processes affected by this gland. The effects caused by the

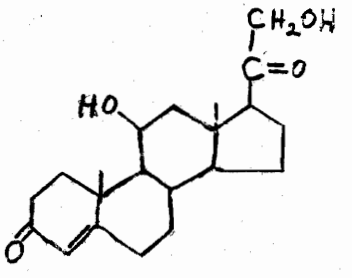
injection of large amounts of the cortical extracts mentioned above further emphasized the importance of the regulatory role of this tissue at many sites.

In the early 1930's, therefore, adrenal cortical physiology was not in a well-defined state. Detailed information concerning the agent or agents responsible for the functions of the adrenal cortex was not forthcoming in the relatively crude experiments described above. The lack of standard preparations, the shortage of pure substances, and the relatively small amounts of expensive extracts available made extensive clinical trials and basic research experiments difficult or even impossible.

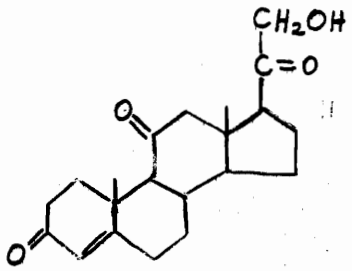
Beginning in 1937, several groups of investigators, notably Kendall, Pfiffner, Wintersteiner and their associates in the United States and Reichstein and his colleagues in Switzerland, isolated from adrenal extracts crystalline compounds with marked adrenal cortical activity. The efforts of these workers, combined with the accomplishments of other groups, have resulted in the isolation of approximately 30 crystalline compounds during the past 20 years. Of the steroids isolated from the adrenal, six have biological activity that mimics one or more actions of whole adrenal extract. These compounds are the following:



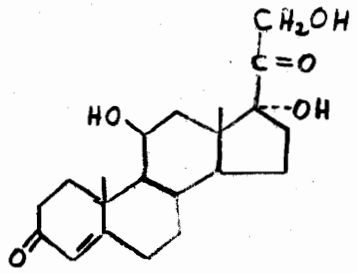
Desoxycorticosterone



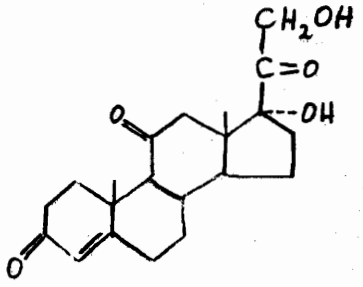
Corticosterone
(Compound B)



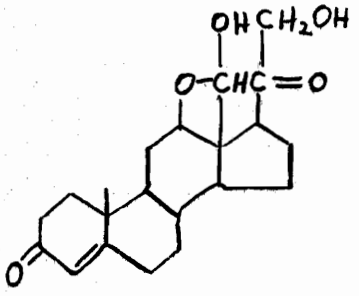
11-dehydrocorticosterone
(Compound A)



17α-Hydroxycorticosterone
(cortisol; hydrocortisone)
(Compound F)



11-Dehydro-17-α-hydroxycorticosterone
(cortisone)
(Compound E)



Aldosterone

17-Hydroxy-11-desoxycorticosterone (Substance S of Reichstein) has also been isolated but in smaller amounts than the six listed above.

All six of the compounds whose formulae appear above have been found in the effluent of perfused adrenal glands of various species. Corticosterone, 17-hydroxycorticosterone, cortisone, 11-dehydrocorticosterone and desoxycorticosterone have all been isolated from beef adrenals stimulated with ACTH (Hecter et al., 1951). A compound that was probably aldosterone was isolated from a calf adrenal perfusate (Rosemberg et al., 1956). The determination of steroid concentrations in peripheral blood has led to somewhat similar findings, although metabolic transformations make steroid levels in blood a less accurate indication of the actual secretory products of the adrenal.

The proportions of the various steroids secreted vary from species to species. For example, the studies of Bush (1951) upon the peripheral blood of several animal species found the following major variations. Dog and cat adrenals secreted mainly hydrocortisone, but small amounts of cortisone and 11-dehydrocortisone were found. The ferret produced approximately equal amounts of hydrocortisone and corticosterone, whereas the rat and the rabbit secreted nearly all corticosterone. In the beef adrenal, Pfiffner (1942) reported the proportions given in Table 1. In the human adrenal cortex, the steroid concentrations have been determined by Neher (1958), and are reported in Table 2.

All the steroids listed above have been synthetized, at least in some measure. The first compound to be prepared in vitro was desoxycorticosterone (Sterger and Reichstein, 1937). It is interesting that this steroid was synthesized one year before it was isolated from adrenal extracts (Reichstein and Von Euw, 1938). Lardon and Reichstein (1943) have made 11-dehydrocorticosterone. A partial synthesis of corticosterone has been published (Von Euw, Lardon, and Reichstein, 1944), and similarly, a partial synthesis of hydrocortisone has been achieved by Wendler et al., (1950). The total synthesis of cortisone (Woodward et al., 1952) and aldosterone (Schmidlin et al., 1955) are also presented in the literature. A detailed summary of synthetic methods for the individual steroids, with the exception of aldosterone, has been published by Woodward et al., (1952). However, only three of the steroids found in the adrenal are available in sufficient quantities for extensive clinical and research studies. Desoxycorticosterone is prepared from stymasterol, a plant steroid, and cortisone and hydrocortisone are prepared from bile acids.

TABLE 1

Adrenal Cortical Hormones in 1000 lb of Beef Adrenals*

		Activity (Dog units)	
In 1000 lb of adrenals			450,000
Corticosterone	350 mg	3,500	
Dehydrocorticosterone	350 mg	3,500	
17-Hydroxycorticosterone	40 mg	200	
17-Hydroxydehydrocorticosterone	500 mg	2,500	
Desoxycorticosterone	15 mg	900	
17-Hydroxydesoxycorticosterone	10 mg	300	
		10,900	439,100

TABLE 2

Adrenal Cortical Hormones in Subjects with Normal Adrenal Function†

Corticosteroid	Cause of Death of Patient from Which Adrenal was Obtained: Traffic Accident	Acute Cardiac Failure	Suicide (hanging)
Cortisol (ug/g adrenal)	2.5-3.9	1.53-2.18	0.13-0.15
Cortisone (ug/g adrenal)	0.04-0.1	0.05-0.39	0-0.17
Aldosterone (ug/g adrenal)	0.05-0.05	0.06-0.38	0-0.07
Corticosterone (ug/g adrenal)	1.1-2.9	2.92-10.6	0.22-0.88

*After Pfiffner, 1942
†After Neher, 1958

The isolation and/or synthesis of these pure compounds allowed the separation of the many effects recorded for crude cortical extracts. It rapidly became apparent that adrenal hormones could be classified according to their metabolic effects (glucocorticoids) or their electrolyte effects (mineralcorticoids). The glucocorticoids are represented in the naturally occurring hormones listed above by cortisol, cortisone, corticosterone and 11-dehydrocorticosterone. The mineralcorticoids are aldosterone and deoxycorticosterone. The glucocorticoids exert profound effects upon carbohydrate, lipid, and protein metabolism; have marked effects upon the number of blood lymphocytes, erythrocytes, and eosinophils, as well as the structure of lymphoid tissue; augment hydrochloric acid and pepsinogen secretion by the gastric mucosa, and trypsinogen secretion by the pancreas; prevent the appearance of an inflammatory response, whether the stimulus is physical, chemical, or bacterial; and maintain homeostasis despite exposure to a similar variety of noxious stimuli, such as, hemorrhage, physical trauma, infectious agents, and noxious chemicals. The mineralcorticoids have a regulatory influence on the relative concentrations of electrolytes, notably sodium and potassium, in the extracellular fluids. It should be stated, however, that the glucocorticoids have some electrolyte effects, and that the mineralcorticoids have some metabolic effects. In addition, the hormones within a group vary in potency with regard to a particular effect normally prominent for the group as a whole. This is illustrated by Table 3 (White et al., 1959).

The concentrations of desoxycorticosterone (DOC) found in the adrenal gland or in peripheral blood are not large enough to account for the effects of this tissue upon electrolyte metabolism. Reichstein and Von Euw (1938)

TABLE 3

Approximate Relative Biological Activities of Certain Adrenal Cortical Steroids in Adrenalectomized Rats, Expressed in Terms of the Activity of 11-Dehydro-17-hydroxycorticosterone (Cortisone) #

Steroid	Life maintenance	Glycogen deposition	Sodium retention*	Muscle-work test†	Growth test	Cold test	Anti-inflammatory activity*
11-Dehydro-17-hydroxycorticosterone (cortisone)	100	100	100	100	100	100	100
Corticostosterone	75	54	255	46	108	9	3
11-Dehydrocorticosterone	58	48	...	32	...	33	0
17-Hydroxycorticosterone (cortisol)	100	155	150	160	219	...	7,680
Deoxycorticosterone	400	0	1,500	5	...	8	0
Aldosterone	...	30	60,000	...	...	...	0

*Adrenalectomized mice.

†Adrenalectomized-nephrectomized rats.

#After White, Handler, Smith, and Stetten (1959).

gave a value of 12.5 mg of desoxycorticosterone per 1000 lbs of glands. However, Kendall (1942) did not find desoxycorticosterone present in 1000 lb of adrenals, and Thatcher and Hartman (1946) were unable to demonstrate its presence in fractions capable of affecting electrolyte concentrations. Hecter et al., (1951) have reported small quantities in the perfusates of adrenal glands in vitro. Farrell et al., (1954) estimated that small amounts (18.6 mg/L blood) of a substance tentatively identified as DOC were secreted by the adrenal gland of the dog following ACTH stimulation. They estimated that the activity of this amount of steroid was much less than that of the aldosterone secreted in the same volume of blood. The data given in Table 1 (Pfiffner, 1942) show that all the compounds known at that time, including DOC but not including aldosterone, account for only 1/40 of the total activity of the gland extract. Thus, it seems that DOC is probably secreted by the adrenal gland in small amounts, but that this secretion is not too important for electrolyte regulation in the normal animal.

Aldosterone is also secreted in very small amounts, but the high potency of these small quantities can account for the activity of the adrenal gland upon electrolyte metabolism. Wettstein (1954) has reported that beef blood contains 0.35 mg per 100 ml, and that the beef adrenal gland contains small amounts, 45-95 mg/kg tissue, of this steroid. Aldosterone concentrations in normal human adrenal tissue are given in Table 2 (Neher, 1958). Simpson and Tait (1955) found a concentration of 0.08 mg/100 ml of this steroid in normal human systemic blood. Farrell et al. (1954) identified aldosterone in the adrenal vein blood of hypophysectomized dogs, and found the sodium-retaining activity of aldosterone to be 48 times that of DOC. Gaunt, Renzi, and Chart (1955) in a recent

review cite papers to show that aldosterone is 30, 85-100 or even several hundred times more potent than DOC in causing sodium retention. Simpson and Tait (1955) have reported conclusive experiments which indicate that aldosterone is the active principle of the amorphous fraction of adrenal extracts. It has not been established unequivocally that all the sodium-retaining activity in adrenal extracts is due to aldosterone, but strong evidence against this hypothesis is lacking. Aldosterone is a natural hormone secreted by the adrenal gland, and the quantity and activity of this secretory product are high enough to be of biological importance in electrolyte metabolism.

A great deal of information concerning the actions of the mineralcorticoids has been obtained by studies utilizing desoxycorticosterone, while comparatively few studies have been completed with aldosterone. This is understandable since desoxycorticosterone was one of the first active steroids isolated from adrenal extracts (Mason, Myers, and Kendall, 1936; Reichstein and Von Euw, 1938), while aldosterone was one of the last (Simpson et al., 1953). Desoxycorticosterone was also one of the first compounds to be synthetized (Steiger and Reichstein, 1937), whereas aldosterone was one of the last to be prepared in vitro (Schmidlin et al., 1955). Desoxycorticosterone has been available in large quantities for approximately 20 years, while aldosterone is even now in short supply. Thus, a unique situation exists in which most of our information concerning the actions of a natural hormone, aldosterone, has been indirectly inferred from studies with a synthetic substitute, DOC.

A comparison of these two compounds indicates some marked quantitative differences, but relatively few, if any, real qualitative differences. Several comparisons are summarized in Table 4 (Gaunt, Renzi, and Chart, 1955).

TABLE 4

SUMMARY OF BIOLOGIC ACTIVITY OF CRYSTALLINE ALDOSTERONE *

Test	Subject	Activity
Na retention	Adx. rat	20-30 X DC
K excretion	Adx. rat	5 X DC
Na^{24} / K^{42} excretion	Adx. rat	120 X DC
Na / K excretion	Adx. rat	30-100 X DC
Water (saline) excretion	Adx. rat	No retention, unlike DC; no diuresis, unlike cortisone
Water diuresis	Adx. dogs; Addisonians	Weak or inactive, like DC
	Adx. rats	Active: > DC; > cortisone; = hydrocortisone
Water intoxication	Adx. rats	> DC; probably > cortisone and corticosterone; = hydrocortisone
Renal functions: rise of GFR & RPF	Adx. rats	> DC; > cortisone; = hydrocortisone
Renal functions: Na retention vs. GFR	Adx. rats	Na retention without elevation of GFR
Substitution therapy	Adx. dogs	12-25 X DC; 500 X hydrocortisone
Substitution therapy	Addisonians	Maintained with 100-200 µg./day; 20-30 X DC
Electrolyte balance	Man	Caused Na and water retention; weight gain
Na / K of saliva	Man	Depressed by 10-20 µg.
Reduced permeability to Na and K	Rat diaphragm	> 10 X hydrocortisone
Cold stress	Adx. rat	= or > cortisone
Reduction of eosinophil count	Adx. mouse	1/3 cortisone
Liver glycogen	Adx. mouse	1/3 cortisone; 30 X DC
ACTH suppression	Rat	1/3 cortisone; 8 X DC
Antagonism to allergic shock	Adx. guinea pig	Weak or inactive, like DC
Granuloma growth -- cotton pellet	Adx. rat	Inactive or stimulatory, unlike other corticoids
Granuloma pouch -- air pocket	Adx. rat	Inhibited cortisone less than DC
Blood pressure	Adx. rat	Maintains B.P. in adrenal insufficiency; not hypertensive
Blood flow in femoral arteries	Adx. cat	Maintains femoral flow response to epinephrine & histamine, unlike other corticoids
Inhibition of acetylcholine	Isolated frog heart	Inactive, unlike other adrenal & sex steroids
Enzyme systems	Rat	Slight differences compared with DC & other corticoids
Maintenance of lactation	Adx. rat	20 X hydrocortisone

*After Gaunt, Renzi, and Chart (1955).

Most of the comparisons substantiate the claim that aldosterone differs from DOC only quantitatively in its spectrum of effects.

Many claims that indicate a qualitative difference between the two steroids are based on experiments concerned with the excretion of water and/or saline loads. Desaulles (1958) imposed hypotonic, isotonic and hypertonic saline loads upon animals treated with either DOC or aldosterone. Aldosterone greatly enhanced the impaired diuretic response of animals submitted to a hypotonic (water) load. Under increasing salt levels, aldosterone did not modify the diuretic response of the animals, while under hypertonic salt loads it actually induced marked water retention. DOC did not modify the impaired urinary excretion of water-loaded animals. However, it produced a definite urinary retention under an isotonic salt load, and induced a slight diuresis under hypertonic salt loads.

Discrete differences upon sodium and potassium excretion were also observed under the same conditions. Aldosterone exhibited a pronounced sodium-retaining action which diminished in intensity as the salt load was increased. DOC exerted the same effect, but its retentive action diminished more rapidly than in the case of aldosterone. Aldosterone exerted an enhancing effect upon potassium excretion that exhibited an inverse relation to the sodium pattern. Under a water load, aldosterone exerted a marked stimulation of the impaired potassium excretion; under salt loads of increasing concentrations the stimulated potassium excretion diminished rapidly. DOC did not modify the impaired potassium excretion under a water load. During the administration of an isotonic solution the excretion of potassium was very high, but the potassium excretion showed only mild stimulation when a hypertonic salt solution was given.

The experiments cited above must be repeated and confirmed before any real difference between aldosterone and DOC can be considered unequivocal.

Gross and Lichtlen (1958) claimed to have found certain differences between the two drugs, but a close inspection of their data reveals that the differences are probably quantitative and not qualitative. It is interesting to note, however, that very high doses of aldosterone did not lead to the polydypsia and polyuria that occur with overdosages of DOC.

Two other reported differences have been the lack of edema formation and the absence of hypertension following aldosterone treatment, while DOC administration frequently causes both symptoms to appear. More recent studies indicate that doses of aldosterone higher than the usual maintenance dose can cause edema (Gross and Schmidt, 1958) and hypertension (Gross and Lichtlen, 1958). Hypertension is also present in primary aldosteronism (Conn and Louis, 1956), and edema may also occur in this clinical entity (August, Nelson, and Thorn, 1958).

Aldosterone is unique with respect to clinical problems, since high aldosterone levels in blood and urine of patients have been reported, while the DOC has not been found in similar patients. A disease called primary aldosteronism is described in which large quantities of aldosterone are secreted by adrenal neoplasms (Conn and Louis, 1956); no such syndrome has been described for DOC. High concentrations of aldosterone are found in many edematous patients, e.g., patients with congestive heart failure and liver cirrhosis; DOC has not been isolated in such situations.

In summary, the long history of the effects of the adrenal gland upon electrolyte metabolism seems to have reached another, if not the final, milestone with the isolation and identification of aldosterone. From the crude ablation experiments to present researches with a crystalline

steroid of complex structure, the road has been difficult and full of pitfalls. Perhaps the most fortuitous event of the whole journey was the discovery and synthesis of desoxycorticosterone. The wealth of research with this drug, which is probably not a natural hormone of any importance, made possible the identification of the natural mineralcorticoid aldosterone and resulted in a new era of understanding in electrolyte physiology as it relates to the adrenal gland.

Aldosterone is almost certainly the natural hormone responsible for the profound effects of the adrenal gland upon electrolyte metabolism. Its availability is opening new vistas of research and the clinical treatment of adrenal insufficiency. Desoxycorticosterone, however, is still an important compound and will continue to occupy a choice place in the laboratories of research workers concerned with electrolyte studies.

II. STATEMENT OF THE PROBLEM

Desoxycorticosterone affects electrolyte concentrations in many tissues of the mammal. Sodium and potassium changes have been studied in the skin (Green, Reynolds, and Girerd, 1955; Davis, Bass, and Overman, 1951), cardiac muscle (Darrow and Miller, 1942; Green, Reynolds, and Girerd, 1955; Muntwyler, Mautz, and Griffin, 1944), skeletal muscle (Miller and Darrow, 1941; Ferrebee et al., 1941; Harkness et al., 1942; Muntwyler, Mautz, and Griffin, 1944), bone (Overman, Davis, and Bass, 1951; Woodbury, 1953; Sweet, Levitt, and Hodes, 1958), liver (Davis, Bass, and Overman, 1951; Woodbury, 1953; Darrow and Miller, 1942), aorta (Daniel and Dawkins, 1951; Tobian, 1956), stomach smooth muscle (Daniel and Daniel, 1955; Green, Reynolds, and Girerd, 1955), spleen (Green, Reynolds, and Girerd, 1955; Davis, Bass, and Overman, 1951), cerebral cortex (Woodbury and Davenport, 1949; Timiras, Woodbury, and Goodman, 1954), tendon (Sweet, Levitt, and Hodes, 1958), ileum (Davis, Bass, and Overman, 1951), lung (Davis, Bass, and Overman, 1951), and testis (Cole, 1950). Most of these results can be summarized by stating that DCA, in general, increases tissue sodium and decreases tissue potassium.

Desoxycorticosterone (DCA) also affects the electrolyte ratio in many body excretions. DCA has been shown to decrease the sodium-potassium ratio in saliva (Prader, et al., 1955; Frawley, 1946) and sweat (Conn, 1949). The reabsorption of sodium and the excretion of potassium by the kidney are increased by this drug (Thorn and Emerson, 1940; Thorn, 1942; Roberts and Pitts, 1952; Pitts, 1952; Roberts and Randall, 1955; Howell and Davis, 1954). The enhancement of sodium reabsorption and potassium excretion via the digestive tract has been satisfactorily demonstrated (Stein and Wertheimer, 1941; Berger, Quinn, and Homer, 1951; Davis and Howell, 1953; Howell and Davis, 1954; Woodbury, 1953). In addition,

DCA also markedly increases the water content of mammary glands, an effect that may be related to electrolyte shifts (Folley, 1952).

Desoxycorticosterone also exerts effects upon other cations and anions. Changes in hydrogen and bicarbonate ion concentrations have been detected in plasma (Lecoq, 1952; Seldin, Welt, and Cort, 1956; Darrow, Cooke, and Coville, 1953), urine (Giebisch, MacLeod, and Pitts, 1955; Roberts and Pitts, 1953), as well as in skeletal muscle (Darrow, Cooke, and Coville, 1953; Cole, 1953; Muntwyler, Mautz, and Griffin, 1944; Harkness et al., 1942). Amino acids which are cations at physiological pH's are also affected by DCA. In particular, lysine concentrations in skeletal muscle are increased after treatment with this steroid (Iacobellis, Muntwyler, and Dodgen, 1956; Eckel, Norris, and Pope, 1958).

There is no question, considering the information presented above, that DCA has marked effects upon the electrolyte, acid-base, and amino acid metabolism of animals and man. There are questions, however, concerning the mechanisms and the interrelationships of these distortions.

Many workers believe that the effects of DCA upon renal tubular absorption and excretion are the primary events in a chain of related changes and that all fluid and/or tissue electrolyte variations are caused by these renal effects. Evidence supporting this thesis can be categorized as (a) data indicating a direct effect of DCA upon tubular functions and (b) experimental results which show that these direct renal effects can cause tissue electrolyte distortions. A direct effect of DCA upon renal tubular function, category (a) above, is supported by the following experiments. Maximum tubular sodium reabsorption occurs after DCA treatment, without significant alterations in glomerular filtration rate (GFR) or renal plasma flow (RPF) (Roberts and Randall, 1955;

Garrod, Davies, and Cahill, 1955). DCA also increases renal tubular bicarbonate reabsorption without changing GFR (Giebisch, MacLeod, and Pitts, 1955). However, Davis and Howell (1954) and Guadino and Levitt (1949) have demonstrated that DCA increases both GFR and RPF. The intravenous infusion of DCA also increases GFR (Sartorus and Roberts, 1949). But in all these experiments, sodium reabsorption was increased. The increased filtration of sodium indicated by the increased GFR is difficult to reconcile with its increased reabsorption without postulating a direct effect of DCA upon the renal tubule to increase sodium reabsorption. The increased GFR and, in some cases, RPF reported in these data are probably secondary to sodium retention and extracellular fluid volume expansion, as indicated by the experiments of Roberts and Pitts (1952). Direct effects of DCA upon renal arterioles have been reported (Friedman, Polley, and Friedman, 1948). However, these effects were observed with high doses of DCA administered for 42 days. The data, therefore, do not necessarily contradict experiments which indicate a direct effect of DCA upon the tubular but merely indicate that DCA can exert effects at other sites. DCA given to dogs made anuric by uretral ligation had no effects upon plasma electrolytes (Winkler, Smith, and Hoff, 1942). This experiment is interpreted by the authors as indicating an effect of DCA only upon the tubule, since DCA causes plasma sodium and potassium changes in normal animals having functional tubules.
It is interesting to note that in a similar ureteral ligation experiment, namely, the stop flow technique of Malvin, Wilde, and Sullivan (1958), aldosterone, the natural hormone whose electrolyte effects are mimicked by DCA, causes a decrease in the lumen concentration of sodium in the distal tubule of the adrenalectomized dog (Vander et al., 1958).

This type of experiment has been interpreted as evidence for a direct action of mineralcorticoids upon the renal tubule. Although DCA administration probably would yield the same results as those seen with aldosterone, no definite conclusions concerning this steroid can be reached until DCA is actually studied. Further, and even more important, the criticisms that have been advanced against the stop flow technique make it mandatory that any conclusion arrived at from data obtained by this method must remain, at best, a tentative one (Pitts et al., 1958; Bradley and Wheeler, 1958). Pitts (1951, p. 40), in summarizing the evidence available at that time said, "Every bit of evidence that I know is consistent with the view that there is a primary effect of adrenal hormones on the tubular cell to increase their absorption of sodium and that this effect occurs promptly." Thus, it seems that the evidence for a direct effect of DCA upon the tubule is unequivocal, at least as far as sodium reabsorption is concerned. Several of the studies cited above were concerned with the effects of DCA upon renal potassium excretion (Howell and Davis, 1954; Giebisch, MacLeod, and Pitts, 1955). These studies led to the conclusion that DCA enhanced potassium excretion by a direct effect.

Evidence has accumulated which indicates that the renal effects of DCA can account for the tissue and/or body fluid distortions observed with this drug, category (b) above. First, tissue distortions have been shown to be reduced if renal effects of DCA are prevented. Seldin, Welt, and Cort (1956) have shown that DCA administered to rats on either low or high potassium intake had no effects on muscle potassium if sodium intake was rigidly restricted. This rigid restriction of sodium has been shown to prevent the renal loss of potassium even during DCA treatment (Howell and Davis, 1954). The reabsorption of bicarbonate

induced by DCA is also dependent upon adequate sodium intake (Giebisch, MacLeod, and Pitts, 1955). These authors showed that a low sodium intake prevented a DCA-induced renal potassium loss and that this lack of potassium excretion prevented bicarbonate reabsorption. Conway and Hingerty (1953) demonstrated that muscle sodium is decreased subsequent to DCA administration on a low-sodium diet, as contrasted to the classical increase in muscle sodium induced by DCA in intact animals fed normal diets. Both plasma and muscle potassium losses were reduced in this experiment when the animals were fed low-sodium diets. Although urine values for potassium were not reported, it is not unreasonable to assume that the low-sodium diets prevented the renal loss of potassium usually caused by DCA and that this lack of potassium depletion was reflected in the muscle electrolyte changes.

The interrelationship of renal and tissue effects of DCA is also indicated by replacement experiments. The muscle distortions of sodium accumulation and potassium depletion caused by DCA are corrected, in part, by the administration of potassium to the animals (Muntwyler and Griffin, 1951). Further evidence along this line is supplied by Ferrabee et al. (1941) who showed that the simple expedient of allowing DCA-treated dogs to drink KCl prevented potassium depletion and the sodium accumulation usually caused by this steroid.

The quantitative importance of the effects of DCA upon the intestinal tract to promote potassium excretion and sodium retention has been determined. Woodbury (1953) has shown that the DCA-induced sodium accumulation and potassium depletion of skeletal muscle still occur in nephrectomized rats. This study also demonstrated that significant potassium losses were taking place in the intestinal tract.

Other groups have pointed out the effects of DCA upon gastrointestinal potassium excretion (Davis and Howell, 1953; Howell and Davis, 1954), as well as the marked effect of this steroid upon sodium retention (Berger, Quinn, and Homer, 1951). However, in only one experiment were the tissue and gastrointestinal electrolyte changes measured simultaneously (Woodbury, 1953). DCA effects upon electrolyte ratios of sweat, saliva, etc., are probably not quantitatively important insofar as tissue electrolyte and acid-base disturbances are concerned.

An imposing collection of miscellaneous results indicates that DCA can directly affect tissue electrolyte ratios. DCA can increase inulin space (extracellular fluid volume) without affecting H_2O space (total body water) or plasma volume (Guadino and Levitt, 1949). An interpretation of these changes is that the steroid has a direct effect upon the intracellular-extracellular water ratio which is not secondary to renal sodium retention. Confirmatory experiments have been done on dogs during enforced hydration (Cheek and West, 1956). Changes in water distribution following DCA administration are not surprising, because the brilliant series of papers by Swingle and coworkers (1934 a, 1934 b, 1936, 1937) demonstrated that (a) animals given cortical extracts could increase blood volume even during enforced hydration; (b) cortical extract can relieve adrenal insufficiency and increase serum sodium and chloride in dogs fed low sodium diets; and (c) cortical extracts increase plasma sodium, chloride and H_2O in water intoxicated animals. The increased survival time of adrenalectomized-nephrectomized animals following DCA treatment, as well as the protection DCA offered against water intoxication symptoms in adrenalectomized rats, was explained by speculating that DCA mobilized cellular water into the

extracellular fluid. That is to say, a direct effect of DCA upon tissue was observed even in the absence of the kidney (Bernie, Eversole, and Gaunt, 1948). Barnett and McNamara (1949) found that DCA could decrease high plasma potassium in a male infant suffering from adrenal insufficiency without increasing renal potassium excretion.

The increase of extracellular sodium observed during the treatment with DCA of dogs with adrenal insufficiency was too large to be explained by the retention of administered sodium (Flanagan, Davis, and Overman, 1950). In a human patient, Lüft and Sjogren (1952) found that exchangeable sodium increased,although the patient was in negative sodium balance. They argued that DCA can increase extracellular fluid sodium without the renal effect of sodium retention; or, in other words, DCA has a direct effect to increase extracellular fluid sodium. In a classic paper, Woodbury (1953) unequivocally demonstrated by direct tissue analysis that DCA could exert significant effects upon cerebral cortex, bone, liver, and skeletal muscle of nephrectomized rats.

The changes in tissue acid-base balance caused by DCA have not been adequately studied. Firstly, the number of actual measurements of changes in tissue hydrogen and bicarbonate ion concentration associated with DCA treatment is very small. In fact, no tissue measurements have been done in experiments in which DCA administration was the only variable. Most tissue measurements have been made on animals that have been made potassium-deficient by a <u>combination</u> of DCA injections and a low potassium diet (see Muntwyler and Griffin, 1951; Darrow, Cooke, and Coville, 1953). Secondly, almost all other studies infer tissue changes from changes in the acid-base picture of the blood. The basis for this

extrapolation is the similarity of DCA treatment and dietary potassium deficiency upon blood pH and blood bicarbonate concentrations. Animals feeding upon low-potassium diets and animals treated with DCA both exhibit a hypokalemic alkalosis. In addition, indirect evidence (Cooke et al., 1952) and a direct measurement of tissue pH in the potassium-deficient animal (Gardner, Machachlen, and Berman, 1952) indicated a cellular acidosis. It has been assumed that DCA causes a cellular acidosis, because DCA administration also induces a potassium deficiency. Considering that the cellular acidosis associated with potassium deficiency has been seriously challenged (Eckel, Botschner, and Wood, 1958), inferred pH changes in muscle become even more hazardous.

On the other hand, there is little evidence to support the idea that DCA has a direct effect upon tissues to cause acid-base distortions. The effects of DCA upon carbohydrate and fat metabolism are minimal. However, effects such as the inhibition of citrate synthesis reported by Cochran and DuBois (1952) could affect carbon dioxide production and thus affect tissue pH. The possibility exists that hydrogen and sodium ions are pumped from the muscle cell by the same carrier mechanism (Woodbury, 1956; Harris, 1956; Swan and Kossman, 1958). DCA may possibly affect tissue pH by enhancing the active transport of both ions, since DCA has been reported to enhance the transport of sodium (Woodbury, 1958).

The direct effects, that is, those not associated with a potassium deficiency, of DCA upon tissue amino acids have been determined only in brain. Vernadakis (1957) has shown that DCA affects brain amino acids in 6-hour experiments in which significant potassium losses did not occur. The possibility that DCA could exert a direct effect upon

skeletal muscle has not been considered.

Therefore, the investigation, the results of which are reported in this dissertation, was initiated to study the effects of DCA upon tissue electrolytes, amino acid concentrations, as well as acid-base balances, in an experimental situation in which the loss of body potassium was prevented. The experiments were performed upon the functionally eviscerated rat in which the lack of renal function and the absence of the gastrointestinal tract prevented potassium depletion when DCA was administered.

It was hoped that such an approach would yield more definite information on the direct effects of steroids on tissue, and that some clarification of the possible interrelationships between changes in electrolyte and amino acid concentration and changes in acid-base balance in tissues would result. Both problems are important and interesting in the field of mineralcorticoid physiology.

III. GENERAL METHODS

A. Evisceration

The animals used in the DCA experiments were functionally eviscerated by removal of the entire digestive tract, by bilateral adrenalectomy, and by functional nephrectomy which consisted of removal of the kidneys from their capsules and tying of the renal arteries (Russell, 1942). A complete evisceration, including removal of the liver, a two-step technique requiring 21 days (Ingle, 1949), was not attempted since no effect of the liver upon the results of the experiments was anticipated, and since the absence of the liver with its glycogen stores makes the problem of maintaining blood glucose levels more difficult. A modification of the functional evisceration of Russell in which circulation through the viscera is prevented without removal of the organs (Depocas, 1958) was used for some experiments. Animals prepared by this method survived for only two hours, and so were useless for the six-hour treatment schedules.

The functionally eviscerated animals became hypoglycemic, since liver function was decreased and the pancreas had been removed (Russell, 1942). However, administration of 20% glucose solution in the amount of 1.5 ml/2 hours/rat helped maintain the blood glucose and increased survival time from approximately 2 hours to 24 hours. The animals had little control over their body temperature and rapidly assumed a temperature which was one or two degrees above ambient room temperature. No attempt was made to control the temperature variable precisely, but animals were heated with lamps when room temperature fell below 23° C.

B. Analytical

Sodium and Potassium. These ions were determined with an internal lithium standard flame photometer (White, 1952). The preparation of samples for analysis was carried out as described by Hald (1947).

Chloride. Plasma chloride analyses were performed in some experiments by the mercuric nitrate titration of Schales and Schales (1941). The recently developed amperometric titration of chloride with silver ion (Cotlove, Tromtham, and Bowman, 1958) was employed in later experiments. By this method, smaller aliquots were more rapidly and easily analyzed. Data from the two methods were not separated in the presentation of experimental results, since chloride values obtained by both methods were in good agreement. Tissue chloride concentrations were determined by the alkaline digestion modification (Sunderman and Williams, 1933) of the method of Van Slyke (1924).

Water. Tissue samples were placed in tared glass bottles, and dried for 4 days at 110° C. The percentage H_2O was determined by the difference between wet and dry weights. Plasma water was determined by drying an aliquot of plasma in a platinum crucible overnight at 110° C.

Fat. Tissue fat was determined by the weight loss of the tissue after 3 extractions with ethyl ether (Hastings and Eichelberger, 1937).

Carbon Dioxide. Plasma total carbon dioxide concentrations were measured by the manometric method of Van Slyke and Neill (1924). Some analyses were carried out by a modification of the chemical method described in Appendix 1, below. Tissue total carbon dioxide concentrations were determined by two methods. The classical manometric method of Danielson and Hastings (1930) was used during the earlier parts of this work. However, reproducible results were almost impossible to obtain, since the method required exposure of the tissue in acid solution

to the atmosphere. In addition, some 20-30 minutes were required for the analysis of one sample. Therefore, a chemical method for tissue carbon dioxide was developed. The method is described in detail in Appendix 1. The majority of the carbon dioxide results presented below were obtained with the chemical method.

Amino acids. Muscle samples were analyzed by paper chromatography methods suggested by Block (1957). The muscle samples were placed in 12% trichloroacetic acid in an ice bath at 0° C and kept frozen until extraction. The samples were homogenized, centrifuged, and the supernatant extracted three to six times with an equal volume of ether in order to remove the trichloroacetic acid and fats. The remaining aqueous phase was evaporated to dryness in a water bath at 37° C. After evaporation, the residues were taken up in an equal weight of water. Samples of 4 to 32 λ were used for paper chromatographic analysis. Whatman No. 1 filter paper was used in sheets of 23 X 28 cm for ascending chromatographic development. The chromatograms of the test samples were run simultaneously with mixtures of amino acids in known amounts. The chromatography chambers were commercial Chromatocabs (Research Specialties Co., Berkeley, California). A 4:1 mixture of phenol (80% distilled over aluminum and sodium bicarbonate) - water was used for development in the first dimension, and a 11:5:4 mixture of 2,6-lutidine (90%)-ethanol(95%)-water, was used for the development in the second dimension. After each development, the papers were dried overnight at room temperature. The papers were then dipped in a solution of ninhydrin (0.25%, in a solvent 95% ethanol containing 2% acetic acid) and dried for 18 hours at room temperature. The amino acids on the paper appeared as colored spots which were sufficiently stable and dense to permit determination of their

optical density. A Welch Densichron (W. M. Welch Mfg. Co., Chicago, Illinois) was employed to read the maximum optical densities of the amino acid spots. Amino acid concentrations were calculated by comparing optical densities of unknown spots with optical densities of spots obtained by similar treatments of known amounts of pure amino acids.

pH determinations. Blood pH was determined anaerobically by means of a glass electrode standardized with commercial buffer (Beckman Instruments, Inc., Fullerton, California) at pH 7.0. All determinations were made with a Cambridge Research Model pH Meter. The pH of a sample was determined at room temperature and corrected to 37° C by the method of Rosenthal (1948).

C. Calculations

Electrolyte distributions in tissues were calculated by the method of Hastings and Eichelberger (1937). Bicarbonate distribution and intracellular pH were calculated as described by Wallace and Hastings (1942). Some discussion of these calculations is warranted, since many conclusions derived from the experiments below are based upon data obtained from these numerical manipulations.

The basic assumptions upon which Hastings and Eichelberger based their ion distribution calculations were:

1. Water moves freely across the barrier between the blood plasma and the extracellular phase, and across the barrier between the extracellular and intracellular phases. This results in osmotic equilibrium between all phases of the system.

2. Inorganic ions and small molecules move freely across the barrier between plasma and the extracellular phase, but large molecules,

notably the proteins of the plasma, do not move across this barrier. This selective permeability to molecules causes an unequal distribution of inorganic ions which is determined by the Gibbs-Donnan distribution law.

3. Under normal conditions, inorganic ions, as well as large molecules, do not move across the barrier between the extracellular and intracellular phases of muscle. This condition of functional impermeability exists only as long as the normal metabolic state of the muscle is maintained. When metabolic processes cease, the barrier becomes permeable both to inorganic ions and to proteins.

4. Under normal metabolic conditions the intracellular phase of muscle contains no chloride.

Each of the above assumptions could be discussed as to its validity, but the assumption most often questioned is the fourth one, which states that all muscle chloride is extracellular. Manery (1954) has summarized evidence supporting the statement that all chloride is extracellular as follows: (1) Whole muscle in situ contains a very small quantity of chloride. Since the extracellular components of muscle have such a high chloride concentration, it is impossible for a large amount to exist inside the fiber. (2) Histological examinations of muscle areas not occupied by muscle fibers correspond to calculated chloride space. (3) Muscles in isotonic sucrose rapidly lose all their chloride. (4) The chloride content of muscle is directly proportional to the chloride concentration of the solution in which the muscle is equilibrated. This is true for frog muscle in vitro and mammalian muscle in vivo. (5) Direct histochemical procedures applied to frozen dehydrated sections of muscle reveal no chloride, except in an occasional damaged

fiber. (6) Direct chemical analysis of isolated muscle fibers shows no chloride present inside the fiber. (7) The chloride space agrees quite well with other measurements of extracellular volume, e.g., bromide and iodide space determinations. In addition to the evidence cited by Manery in support of chloride being distributed only extracellularly, another type of experiment was recently reported which substantiates this proposal. The amount of extracellular fluid in the rat was determined by correcting total body chloride and sodium for the amount of these ions outside the extracellular fluid (Cheek, West, and Golden, 1957). The close agreement of extracellular volume determined by sodium and by chloride was cited as evidence that chloride distribution is a valid measure of this body compartment.

Evidence against the extracellular position of all the chloride is as follows: (1) The inulin space of muscle is smaller than the chloride space. (2) Yannet and Darrow (1940) found evidence of nondiffusible chloride amounting to 1 mM/100 gm fat-free solids. (3) Various criticisms of the analytical methods for chloride. (4) Wilde (1945) has demonstrated an entrance of chloride into fibers with potassium. Conway (1957) has interpreted these data as showing that chloride freely permeates skeletal muscle and is distributed according to a Donnan equilibrium. (5) Use of chloride space in <u>all</u> situations gives unreasonable results, such as negative intracellular sodium values (Woodbury, 1956) and intracellular concentrations of potassium and magnesium in skin cells (Harrison, Darrow, and Yannet, 1936) quite different from those found in other tissues. (6) Manery (1937) approached the problem from another viewpoint. She assumed that the extracellular phase of tissue water is an ultrafiltrate of serum, and that electrolyte concen-

trations of the ultrafiltrate are predictable from electrolyte concentrations in serum by application of the Gibbs-Donnan distribution law. She found that some tissue cells, e.g., tendon, muscle sheath, and skin, appeared to contain so much solid material that only 40 per cent of the cell was water. Some calculations even resulted in negative intracellular water concentrations. She concluded that these unreasonable results are explained by high concentrations of intracellular chloride.

What, therefore, can be decided from the above information, without going into detail concerning each pro and con argument? Firstly, some tissues definitely contain intracellular chloride, for example, red blood cells (Bernstein, 1954) and connective tissue (Manery, 1937). No such unequivocal evidence exists for skeletal muscle. However, even if chloride ions freely penetrate muscle cell membranes, the intracellular concentration must still be quite low. The extracellular-intracellular ratio of chloride is determined by the membrane potential of the cell if chloride ions are freely diffusible and not actively transported. The ratio determined by a membrane potential of 90 mV- is 35 to 1, extracellular chloride to intracellular chloride. Therefore, water distribution would be in error by approximately 1/35, or 3%, if the distribution calculations were based upon the assumption that all chloride is located extracellularly. Secondly, many of the objections to chloride space being used as a measure of extracellular space can be softened with the proper experiment. For example, Cotlove (1954) demonstrated that inulin and chloride space are equivalent when enough time is allowed for the inulin to penetrate connective tissue. Nichols et al. (1953) found a reasonable agreement between inulin and chloride

space when tissues were corrected for the connective tissue content of the samples. Thirdly, chloride space can accurately indicate extracellular fluid changes, even though some chloride might be located intracellularly. Caster (1959) showed that raffinose space, one of several substances tested which could measure extracellular fluid volume, best predicts the changes expected when extracellular fluid volumes were changed by X-irradiation. Eckel, Botschner, and Wood (1959) demonstrated the agreement of raffinose space with chloride space in skeletal muscle in potassium deficiency studies. In conclusion, it seems reasonable that chloride space is a valid measure of extracellular space in skeletal muscle in several states of imbalance. It should be admitted, however, that little is known about the distribution of chloride in pathological states, e.g., evisceration, or the effect of drugs upon chloride permeability. Therefore, caution should be observed in experimental interpretations, and emphasis should be placed on space changes rather than rigid quantitative interpretations of space sizes.

The calculations for determining the distribution of bicarbonate in muscle are based upon the extracellular location of chloride and the following additional assumptions. (1) The carbon dioxide tension is equivalent to H_2CO_3 concentration, since the equilibrium $CO_2 + H_2O \leftrightarrow H_2CO_3$ is far to the left. (2) The carbon dioxide tension is the same in all body compartments. (3) The muscle cell membrane is impermeable to the bicarbonate ions. (4) Cell carbon dioxide released by acid is derived from bicarbonate or carbonic acid, and not from any bound form of carbon dioxide such as carbamino groups.

Considering these statements in order, the high concentration of

CO_2 with respect to H_2CO_3 proposed in number (1) has been conclusively demonstrated in plasma where the ratio is 1000 CO_2 to 1 H_2CO_3. The second assumption is acceptable in most cases, since extracellular CO_2 tension changes are reflected in cellular CO_2 tension changes almost immediately (Nichols, 1958). However, equal tensions of CO_2 in all body compartments are not observed in all experimental situations. Koch and Woodbury (1958) have presented evidence that acetazolamide prevents the attainment of CO_2 equilibrium between brain cells and the extracellular fluid. They argued that the inhibition of carbonic anhydrase by the drug was responsible for the observed disequilibrium. Such arguments do not apply to skeletal muscle since this tissue contains negligible amounts of carbonic anhydrase (Van Goor, 1940). Therefore, the assumption of equal CO_2 tension with respect to plasma and tissue was considered valid, particularly for skeletal muscle, and this assumption was used throughout the calculations reported below.

The impermeability of muscle membranes to the HCO_3^- ion has been proposed chiefly on the basis of the work of Wallace and Hastings (1942), who showed that large changes in extracellular HCO_3^- resulted in little change in cellular HCO_3^-. In addition, Caldwell (1958) demonstrated that the internal pH of crab muscle fiber measured directly with a glass electrode is not drastically influenced by changes in extracellular HCO_3^-, but increased CO_2 tensions rapidly decreased cell pH. These results supported both the carbon dioxide permeability and the bicarbonate impermeability assumptions. Waddell and Butler (1959) measured the influence of extracellular bicarbonate concentrations upon cell pH by utilizing the weak base dimethyloxazolidine$_{\wedge}^{\text{dione}}$(DMO) to measure cell pH.

They concluded that the muscle cell membrane is impermeable to bicarbonate, since external HCO_3^- was not reflected in cellular pH changes. Hill (1955) argued that a functional impermeability to bicarbonate exists if a hydrogen ion gradient is actively maintained and CO_2 tension is equal in all fluid compartments, both of which are likely in skeletal muscle. That is to say that the penetration of the muscle membrane by HCO_3^- is unimportant if the cellular hydrogen ion and CO_2 concentrations are fixed so rigidly that no changes in the equilibrium $HCO_3^- + H \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$ can occur.

The impermeability of the muscle membrane to HCO_3 has been questioned by Conway and Fearon (1944). In addition, these authors have proposed that all the CO_2 liberated by acid from muscle is not derived from HCO_3^- and H_2CO_3. Since experimental evidence supporting both objections is examined more closely in Section VII, a discussion of the statements above is deferred for the present. However, both assumptions questioned by Conway and Fearon were utilized in all acid-base calculations.

A summary of all calculations used for determining the partition of ions is recorded in Appendix 2.

IV. EFFECTS OF DCA UPON THE ELECTROLYTE METABOLISM OF SKELETAL MUSCLE.

A. Introduction

The effects of DCA upon skeletal muscle electrolytes are well-known. This steroid increases intracellular sodium and decreases intracellular potassium in the intact animal (Harkness et al., 1942; Muntwyler and Griffin, 1951; Miller and Darrow, 1951). Similar effects have been observed in nephrectomized rats (Woodbury, 1953).

The effects of DCA upon muscle are paradoxical when compared with the drug's action at other sites. Woodbury, Timiras, and Vernadakis (1957) have shown that DCA decreased cerebral cortex cellular sodium, and have speculated that the action of this steroid is an effect upon active sodium extrusion. Recently, Pitts (1958) advanced the theory that renal sodium reabsorption is an active process. It is not unreasonable to assume that the renal reabsorption of sodium caused by DCA is an enhancement of this active process. In the isolated frog skin, DCA caused a water movement from outside to inside (Taubenhaus, Fritz, and Morton, 1956). It was proposed that this water shift was an expression of a direct action of DCA upon the active transport of sodium. The large increase in resting potential of frog skin observed by Taylor and Weinstein (1952) when DCA was added to their perfusion bath can be explained by proposing that the drug enhances sodium pumping. Thus, it seems that one mechanism of action of DCA is to enhance the active transport of sodium. Why, then, does skeletal muscle accumulate cellular sodium after DCA treatment, when the sodium pump in muscle is thought to extrude this cation from an intracellular to an extracellular site?

Evidence has been published which indicates that the shift of sodium

into cells during DCA administration, instead of the expected extrusion of this cation, is caused by loss of muscle potassium. A falling plasma potassium concentration causes potassium to move from muscle cells into plasma, since muscle potassium serves as a reservoir for the maintenance of plasma potassium levels. Plasma potassium concentration falls during hormone treatment because of the marked enhancement by DCA of potassium losses via the kidney and gastrointestinal tract. Ferrabee et al. (1941) showed that the addition of KCl to drinking water prevented the usual muscle sodium accumulation observed with the drug. Conway and Hingerty (1953) found that the effect of DCA upon the muscle of an animal maintained on a low-sodium diet is to cause a decrease in tissue sodium. Concomitant measurements upon animals fed high-sodium diets showed that an increase in muscle sodium was observed when DCA was administered. DCA treatment caused a more severe muscle potassium depletion in the animals fed high-sodium diets than in the animals fed low-sodium diets. The failure of low-sodium animals to lose as much muscle potassium as the animals supplied with ample sodium can be explained by the fact that renal loss of potassium caused by DCA is greatly reduced when low-sodium diets are used for animal feeding (Howell and Davis, 1954; Seldin, Welt, and Cort, 1956).

A comparison of the effects of aldosterone and DCA upon the cerebral cortex and skeletal muscle of mice has revealed that DCA decreases cellular sodium in brain, but increases cellular sodium in muscle; however, aldosterone decreases cellular sodium in both muscle and brain (Woodbury and Koch, 1957). Since aldosterone and DCA both probably enhance sodium transport, and since brain and muscle probably contain similar transport systems, some other reason for these anomalous results must be sought.

The quantitative difference in the effects of the two steroids upon potassium metabolism is a probable explanation. The effects of DCA upon renal potassium losses are more pronounced than those of aldosterone (Swingle et al., 1954). For equisodium-retaining dose, DCA is 5 times more potent than aldosterone with regard to renal potassium excretion. Tissue measurements in the work of Woodbury and Koch showed that DCA slightly increased brain cellular potassium concentration while muscle cellular potassium concentration was definitely reduced following DCA treatment. Aldosterone-treated animals had normal intracellular potassium concentrations in both brain and skeletal muscle. Since increased sodium concentrations were observed only in cells losing potassium, additional support is given the hypothesis that muscle cell sodium accumulation following DCA treatment is secondary to a loss of cellular potassium.

The administration of DCA and 1% sodium chloride caused rabbits to exhibit marked abnormalities in their electrocardiographic patterns (Hall, Diserens, and Hall, 1954). These electrocardiographic abnormalities were similar to those of hypokalemia (Howard and Carey, 1949; Roberts and Magida, 1953; Magida and Roberts, 1953). The electrocardiographic abnormalities were reversed by the substitution of 1% potassium chloride for the 1% sodium chloride, even though hormone treatment was continued. Since data indicating that DCA treatment causes cardiac muscle cells to lose potassium and to gain sodium have been reported (Darrow and Miller, 1942), the experiments above suggest that cellular sodium accumulation seen with DCA treatment is secondary to cellular potassium loss.

The possibility of DCA having primary extrarenal actions has been presented in Section II above. The well-known renal and gastrointestinal

effects of this steroid to enhance potassium excretion have been presented in the same section. Therefore DCA was administered to eviscerated rats to detect any direct effects of the steroid that have been masked by total body and/or tissue potassium losses.

B. Methods

Acute experiments. Male Sprague-Dawley rats of various weights were the subjects in all experiments. Uniform weight rats were used in individual experiments, but different size rats were utilized for different studies. Rats weighing approximately 400 grams were employed for most investigations.

The animals were eviscerated (Section III) and divided into two groups. One group, the treated animals, received a subcutaneous injection of 1 ml/rat of a solution containing 20 mg/ml of desoxycorticosterone acetate (Percorten, CIBA) suspended in 0.9% saline containing a few drops of Tween 80. Another group, the controls, were injected subcutaneously with 1 ml/rat of the suspension vehicle.

Each animal received three injections consisting of 1.5 ml of a 20% solution of glucose. The first injection was given immediately following surgery, the next injection was given two hours later, and the last injection was given four hours postoperatively.

Six hours after drug treatment, the animals were anesthetized with ether and killed by exsanguination via the abdominal aorta. Skeletal muscle samples were obtained chiefly from the gastrocnemius; cerebral cortex samples were procured by separating the cortex from the brain stem and midbrain with tweezers. Tissue samples were prepared for analysis by drying 4 days and grinding the samples in a mortar. Brain samples were ground with glass rods in their drying bottles, due to the

extremely small sample size. Plasma for analysis was obtained by centrifuging fresh blood samples at 3000 r.p.m. for 30 minutes.

An identical experiment was performed on intact rats, so that a comparison could be made between eviscerated and normal rats.

Chronic experiments. Rats of the same size and strain utilized in the acute experiments were employed for these studies. The eviscerated rats were not used for chronic studies because of the short survival time of this preparation. The dosage regimen was 5 mg of DCA/rat/day (given as one 0.25-ml injection of the DCA suspension above) for 4 days. No glucose was given any of these rats. The sacrificing and sampling procedure are cited above.

C. Results and Discussion

An effect of DCA to decrease cellular sodium was observed in the eviscerated rat, as shown in Tables 5&6.The effect was not seen in the intact rat treated chronically with DCA; indeed, a cellular sodium accumulation was observed in the intact animal, as presented in Tables 7 & 8. The eviscerated rat lost little cell potassium during the drug treatment, but the intact rat had a marked cellular potassium depletion. Therefore, it appears that DCA exerts a direct effect upon muscle cells to decrease cellular sodium, but that this effect is not seen if severe muscle potassium depletion causes an overwhelming shift of sodium into muscle cells. On the other hand, intact rats treated for 6 hours with DCA did not lose either muscle potassium or muscle sodium during the treatment period, as shown in Tables 9 & 10.

Why, then, does the intact animal not respond to DCA in the same manner as the eviscerated rat when neither preparation exhibits a loss

TABLE 5

Effect of DCA on Total Electrolytes and Water in Plasma, Brain, and Muscle of Eviscerated Rats.[1]

	H_2O	mEq/liter		
PLASMA	%	Na	K	Cl
Controls (7)	91.67 ± .11	134.7 ± 2.6	5.19 ±.17	105.2 ± 1.54
DCA (7)	92.04 ± .095	136.2 ± 2.1	6.80*** ±.058	101.1 ± 1.8
	H_2O	mEq/kg wet tissue		
BRAIN	%	Na	K	Cl
Controls (7)	78.36 ± .21	48.96 ±1.1	107.8 ± 1.4	28.87 ±1.2
DCA (7)	78.62 ± .16	47.59 ± .88	108.9 ± .79	32.42 ±1.3
	H_2O	mEq/kg wet tissue[2]		
MUSCLE	%	Na	K	Cl
Controls (7)	75.12 ± .26	15.90 ± .36	103.3 ± 1.6	8.95 ±.45
DCA (7)	75.41 ± .19	15.31 ± .56	96.6** ± 1.5	11.04* ±.83

[1]Values given are mean ± standard error.

[2]Values given are calculated on a fat-free basis.

Numbers in parentheses ( ) = number of animals reported.

Asterisks in this table and subsequent tables denote statistical significance: *** $p < .001$
** $p = 0.01$-$.001$
* $p = 0.05$-$.01$
None $p > 0.05$

TABLE 6

Effect of DCA on Extracellular and Intracellular Distribution of Electrolytes and Water in Brain and Muscle of Eviscerated Rats.[1]

	Cl space, %		Intracellular concentration					
			H_2O, %		mEq/kg cell water			
					Na		K	
	Brain	Muscle	Brain	Muscle	Brain	Muscle	Brain	Muscle
Controls (7)	24.06 ±1.2	7.11 ±.33	54.38 ±1.4	68.01 ± .46	29.04 ±1.7	8.84 ±.86	195.2 ± 6.0	151.5 ± 2.9
DCA (7)	27.67 ±1.4	9.42** ±.72	50.51 ±1.4	65.81* ± .83	16.82** ±3.0	3.23*** ±.25	212.7 ± 6.0	145.7 ± 2.0

[1]Values given are mean ± standard error.

Numbers in parentheses ( ) = number of animals reported.

TABLE 7

Chronic Effect of DCA on Total Electrolytes and Water in Plasma and Muscle of Intact Rats[1]

	H_2O	mEq/liter		
PLASMA	%	Na	K	Cl
Controls (6)	92.11 ± .12	136.2 ± 2.5	3.87 ±.19	105.1 ± 1.61
DCA (9)	92.02 ± .14	137.8 ± 3.0	2.97** ±.18	102.2 ± 1.52
	H_2O	mEq/kg wet tissue[2]		
MUSCLE	%	Na	K	Cl
Controls (6)	75.07 ± .31	16.11 ± .32	91.95 ±2.1	10.12 ± .62
DCA (9)	75.52 ± .21	20.40*** ± .46	87.07 ±1.4	9.94 ± .72

[1]Values given are mean ± standard error.

[2]Values given are calculated on a fat-free basis.

Numbers in parentheses ( ) = number of animals reported.

TABLE 8

Chronic Effect of DCA on Extracellular and Intracellular Distribution of Electrolytes and Water in Muscles of Intact Rats[1]

	Cl space, %	Intracellular concentration		
		H_2O %	mEq/kg cell water	
			Na	K
Controls (6)	8.42 ±.61	68.6 ± .51	6.13 ±.38	133.7 ± 2.8
DCA (9)	8.48 ±.43	68.5 ± .88	12.64*** ±.61	126.1** ± 2.4

[1]Values given are mean ± standard error.

Numbers in parentheses ( ) = number of animals reported.

TABLE 9

Acute Effect of DCA on Total Electrolytes and Water in Plasma and Muscle of Intact Rats[1]

PLASMA				
	H_2O	mEq/liter		
	%	Na	K	Cl
Controls (5)	91.8 ± .10	136.7 ± 2.9	3.70 ±.20	104.6 ± 1.61
DCA (5)	91.6 ± .12	138.9 ± 3.0	3.13 ±.15	106.4 ± 1.91
MUSCLE				
	H_2O	mEq/kg wet tissue[2]		
	%	Na	K	Cl
Controls (5)	75.06 ± .31	20.30 ± .61	99.61 ±1.4	11.65 ± .38
DCA (5)	75.31 ± .26	20.38 ± .34	99.81 ±1.2	11.98 ± .49

[1]Values given are mean ± standard error.

[2]Values given are calculated on a fat-free basis.

Numbers in parentheses ( ) = number of animals reported.

TABLE 10

Acute Effect of DCA on

Extracellular and Intracellular Distribution

of Electrolytes and Water in Muscle of Intact Rats[1]

	Cl space %	Intracellular concentration		
		H_2O %	mEq/kg cell water	
			Na	K
Controls	9.74	67.3	9.67	147.7
(5)	±.57	± .63	±.32	± 2.7
DCA	9.84	67.2	9.20	148.0
(5)	±.37	± .82	±.48	± 3.10

[1]Values given are mean ± standard error.

Numbers in parentheses ( ) = number of animals reported.

of muscle potassium? Firstly, the eviscerated animals are in a debilitated state. Perhaps the administration of DCA enhances the activity of a sluggish sodium pump, but has little effect upon an extrusion mechanism working at normal efficiency. Secondly, the intact animals have normal adrenal function; the eviscerated animals do not. An antagonism may exist between the normal adrenal secretion and DCA, as discussed by Thorne et al. (1953) with regard to DCA and cortisone. No evidence available suggests that corticosterone, the hormone secreted by the rat adrenal, antagonises DCA. Thirdly, the acid-base picture of the animals is different. The intact rat treated six hours with DCA did not exhibit any detectable acid-base changes. The eviscerated rat shows a marked cellular alkalosis (see below, Section V). If a competition between sodium and hydrogen ions exists for the sodium carrier, and if hydrogen ion concentration is decreased, the cell could pump more sodium and decrease the concentration of this ion in the cell. It is apparent that the unusual nature of the eviscerated preparation makes comparisons difficult.

The decreased cellular sodium caused by DCA has been difficult to confirm. One variable that affects results is temperature. The eviscerated rat has little control of his body temperature and rapidly assumes a temperature 1 or 2 degrees above ambient room temperature (Ingle, Prestrud, and Nezamis, 1950). Some experiments were done in very warm rooms, 31° C.; some were done in comfortable rooms at 23° C. The animals studied in the warm summer months usually showed the most striking cellular sodium depletion. The cellular sodium decrease is also hard to demonstrate in older animals.

The direct effect observed is probably an enhancement of sodium transport. Muscle behaves like brain in the eviscerated preparation, as shown in Table 6. Brain sodium transport has been studied care-

fully and found to be increased by DCA. Therefore, it is reasonable to assume that the muscle transport system would behave in the same way. Conclusive proof will depend upon radioactive sodium flux measurements in muscle.

V. EFFECTS OF DCA UPON SKELETAL MUSCLE ACID-BASE BALANCE

A. Introduction

Potassium is lost from cells of skeletal muscle when total body potassium is depleted by DCA or by extended maintenance of animals upon potassium-deficient diets (Harkness et al., 1942; Muntwyler and Griffin, 1951; Cotlove et al., 1951). Several workers have found that from one-half to two-thirds of the potassium lost from cells is replaced by sodium (Conway and Hingerty, 1948; Cooke et al., 1952). Even when potassium-deficient diets are continued for 135 days, the amount of sodium accumulated by the cell never balances the amount of potassium that has left the cell (Orent-Keiles and McCollun, 1941). Thus, compared to normal muscle, potassium-deficient muscle has an alkali metal cation deficit.

The brilliant papers of Darrow and coworkers showed that a decrease in muscle potassium is associated with a rise in serum bicarbonate (Darrow et al., 1948). Muntwyler et al. (1950) confirmed this relationship, although they qualified their results by indicating that decreased plasma chloride levels, as well as a potassium deficiency, were necessary for the production of an alkalosis. Balance studies by Cooke et al. (1952) demonstrated that hydrogen ions were liberated from skeletal muscle during the administration of potassium salts to a potassium-deficient rat. The appearance of hydrogen ions occurred simultaneously with a correction of muscle sodium accumulation and potassium depletion caused by the potassium-depleting regimen used for animal feeding. Cooke reasoned that if hydrogen came from muscle during potassium replenishment, it could have entered the muscle secondary to a potassium depletion of the tissue. He reasoned further that a shift of hydrogen

ions from plasma or extracellular fluid to the tissue would be consistent with the extracellular alkalosis recorded by Darrow and his colleagues. Thus, he proposed the classic mechanisms pictured below in figures 1 and 2 to explain the hypokalemic alkalosis observed with potassium deficiency, as well as its correction by the administration of potassium.

Other workers, commenting upon the results of balance studies in humans depleted of potassium, have speculated that hydrogen ions move into cells when the cell is depleted of potassium (Black and Milne, 1952; Huth, Squires, and Elkington, 1959).

Determinations of muscle cell pH by the indirect method of Wallace and Hastings (see Section II) have shown that muscle cell hydrogen ion concentrations increase when the cell loses potassium. Gardner, MacLachlan, and Berman (1952) have reported that muscle cell pH decreases from 6.98 to 6.42 when rats were fed a low potassium diet for 40 days. Similar studies by Schwartz and Wallace (1952) yielded values which showed a pH change from 6.98 to 6.62.

Not all studies, however, confirm the cellular acidosis cited above. The data of Darrow, Cooke, and Coville (1953) showed no marked cellular pH decrease in potassium-deficient animals. In fact, a rough calculation of intracellular pH, based upon the data given for the animal group with the greatest extracellular bicarbonate and muscle potassium distortion, showed that cell pH decreased only 0.1 of a pH unit. Even this decrease might be too large, because no data were given which allowed an accurate calculation of pCO_2. Eckel, Botschner, and Wood (1959) found that muscle intracellular pH did not change when muscle potassium was decreased by means of a potassium-free diet. Further, many studies have reported large muscle potassium losses associated with only a

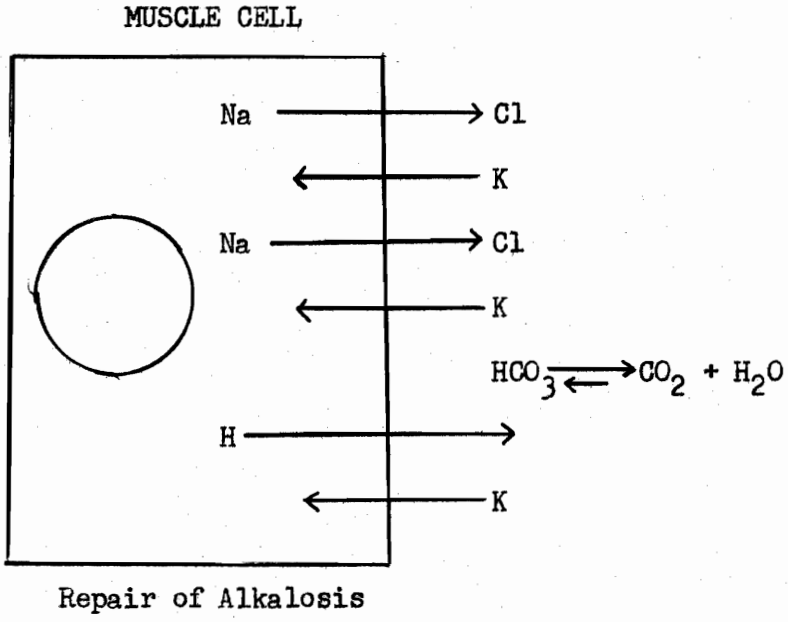


Fig. 1. Cell Transfer Correcting Alkalosis with Administration of Potassium Chloride *

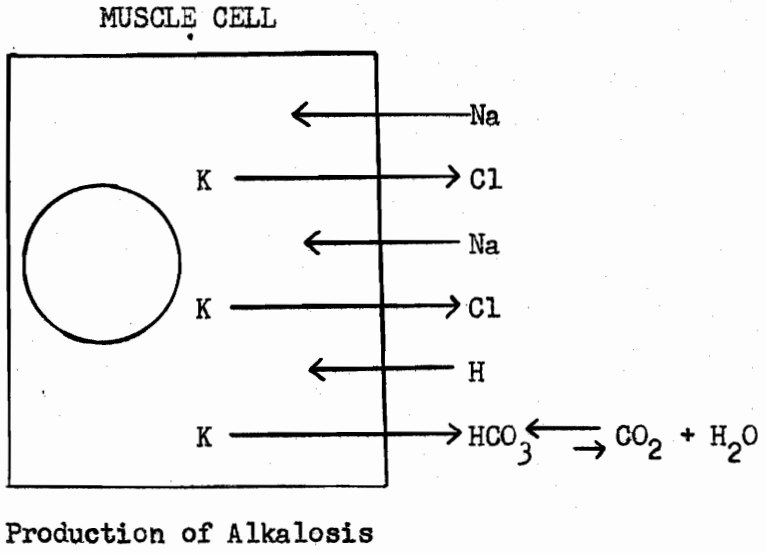


Fig. 2. Cell Transfer Producing Alkalosis in Potassium Loss *

*After Cooke et al. (1952).

minimal extracellular alkalosis (Elkington, 1957; Schwartz and Relman, 1953; Fourman, 1954; Pearson and Eliel, 1951; Schwartz, Levine, and Relman, 1954; Holliday and Segar, 1957).

In many instances, the lack of correlation between potassium loss and acid-base changes has been explained by dietary factors. Animals fed low-sodium diets generally did not have an extracellular alkalosis of marked degree, even when they were fed low-potassium diets (Darrow et al., 1948; Holliday and Segar, 1957). The fact that the animals must lose chloride to exhibit an alkalosis has been pointed out by Muntwyler et al. (1950). In addition, the ratio of sodium to chloride has been shown to be very important. Black and Milne (1952) found an alkalosis associated with a potassium deficiency, but they administered large quantities of fixed cation that was not covered by fixed anion. The large load of alkali contributed to, and perhaps even caused, the alkalosis observed. Carone and Cooke (1953) treated rats with DCA while feeding them a potassium-deficient diet, duplicating in many respects the experiments of Darrow et al. (1948). Carone and Cooke maintained an equimolar sodium to chloride ratio, but Darrow and co-workers had sodium to chloride ratio of 1.5 to 1.0. Carone and Cooke found little elevation of serum bicarbonate; Darrow and his colleagues observed a marked extracellular alkalosis. Moore et al. (1955) summarized several similar observations by stating that potassium loss unaccompanied by excessive sodium administration shows little tendency to cause alkalosis; and that potassium loss complicated by extrarenal salt loss high in chloride, or by the administration of sodium, causes a significant alkalosis and a hypokalemia.

A direct effect of adrenocortical steroids could also be responsi-

ble for the lack of a predictable relationship between potassium losses and acid-base changes in extracellular fluid and tissues. The importance of a direct effect of adrenocortical hormones is indicated by the appearance of high serum bicarbonate values (total CO_2 content of 33-45 mEq/L) in association with increased adrenocortical activity of either exogenous or endogenous origin (Elkington, Squires, and Crosley, 1951; Eliel, Pearson, and White, 1952; Moore et al., 1955). Potassium losses reported in these instances amount to 1-6 mEq/kg. Since larger potassium losses (6-12 mEq/kg) have been reported (Schwartz and Relman, 1953; Elkington, 1957) with only a minimal degree of alkalosis (total CO_2 content of 27-33 mEq/L), it appeared that the more severe alkalosis cannot be attributed to potassium losses alone. Moore and his colleagues (1955) reviewed the literature carefully and concluded that DØCA, ACTH administration or stress (such as trauma or surgery) enhances the severity of potassium deficiency alkalosis. Grollman and Gamble (1959) recently reported that DCA can cause a metabolic alkalosis by a direct effect that is not associated with any decrease in body potassium.

Insofar as muscle is concerned, the work reported above is deficient in two respects. Firstly, most of the information about DCA effects upon muscle acid-base changes has been extrapolated from potassium deficiency experiments. Enough information has been presented above to indicate that differences probably exist. Secondly, muscle pH has not been determined in any experiment in which DCA administration was the only treatment given the animal. All reported experiments include simultaneous low-sodium, low-potassium, low-protein, or low-chloride diets which complicate results. That is to say that the direct effects of steroids

upon muscle acid-base balance have not been considered, with the possible exception of the work of Grollman and Gamble (1959) cited above. Therefore, the experiments reported below were performed to study the direct effects of DCA upon skeletal muscle by doing a complete acid-base analysis of the blood and tissues of the eviscerated rat.

Acid-base measurements could conceivably give some information about another problem concerning muscle hydrogen ion concentrations. Namely, are hydrogen and sodium ions actively extruded from the muscle cell by the same carrier mechanisms? The basis for such a postulate is as follows: Firstly, both ions are actively transported in muscle. The pumping of sodium in skeletal muscle is well known and not subject to question (see Conway, 1957). Hydrogen ion pumping is indicated by the distribution of hydrogen ion against a potential gradient. Extracellular hydrogen ion concentration is 4×10^{-8}; the extracellular pH is 7.4. A reasonable value for the membrane potential of normal skeletal muscle is 90 mV, the inside of the fiber being negative with respect to the outside. If the membrane is permeable to hydrogen ions and if these ions are distributed passively according to the Donnan equilibrium, the following relation holds between external and internal pH's and the membrane potential, at 25° C:

$$\text{Internal pH} - \text{pH of surroundings} = \frac{\text{resting potential (mV)}}{59}$$

At a membrane potential of 90 mV and an external pH of 7.4, the internal pH of muscle is calculated to be 5.9. However, the internal pH is in the vicinity of 7.0 (see Section IV above; Wiercinski, 1955; Caldwell, 1956); or, in other words, the cell is more alkalotic than predicted by theory. The removal of hydrogen ions from the cell to the extracellular

fluid is an explanation for the relative alkalosis of the cell. Secondly, other studies indicate that hydrogen ion-sodium ion competition for a common carrier is a feasible hypothesis. Schoffeniels (1956) has shown that hydrogen ions can inhibit the transport of sodium in frog skin. Conway and Kerman (1955) have found that baker's yeast can transport sodium and hydrogen ions equally well by supposedly the same carrier system. In frog muscle Swan and coworkers (1958) have presented evidence that sodium efflux is an inverse function of intracellular pH.

The known effect of DCA to enhance sodium transport could be used as a tool to study this problem, provided the hydrogen ion changes were large enough to be detected by the available methods. The possibility of some success in clarifying this problem was an added incentive to complete the experiments on muscle acid-base balance outlined above.

B. Methods

The experimental design, the sacrificing procedures, and the sampling techniques have been outlined above in Section III B. However, some special sampling techniques were required for the acid-base samples. The techniques are discussed in the following paragraph.

Blood samples were collected in oiled syringes containing heparin. A fraction of each blood sample was used for a pH determination, as discussed in Section III. Blood pH was determined immediately before any significant lactic acid production by the red blood cells could occur (Peters and Van Slyke, 1938). The remainder of the blood was placed under mineral oil and centrifuged as soon as possible, usually within 30 minutes of sampling time. Plasma samples were separated from red blood cells, and stored under mineral oil in a refrigerator until analysis. Carbon dioxide analyses were performed on plasma samples by

either of the two methods cited above (Section III), within 24 hours of their procurement. The solubility and diffusion rate of carbon dioxide in mineral oilhave been studied by Kubie (1927). His diffusion experiments are difficult to evaluate quantitatively, but a definite loss of carbon dioxide through a mineral oil layer was evident from his results. The validity of results obtained by analyzing plasma samples that had been stored under oil for 24 hours, in view of Kubie's experiments, could be questioned. However, the carbon dioxide content of fresh samples compared to samples that had stood for 24 hours was not significantly different. Tissue samples were collected and analyzed as discussed in Appendix 1. Calculations were completed according to the procedure outlined above in Section III and Appendix 2.

C. Results and Discussion

The acid-base changes in the intact animals treated 4 days with DCA are shown in Table 11. Acid-base changes in eviscerated rats are presented in Table 12 . The data for intact rats treated only 6 hours with DCA are shown in Table 13 . Cell values were not calculated because the observed lack of a significant change in either plasma pCO_2 or tissue total CO_2 would not be commensurate with a change in cell pH. All data in these tables are calculated for individual animals.

The data in Table 11 show that DCA causes an extracellular alkalosis in intact animals. The extracellular alkalosis shown here differs in one respect from the alkalosis associated with potassium deficiency in one important respect. The concentration of plasma H_2CO_3 is reduced in DCA animals, but animals made deficient in potassium by dietary restriction of potassium show an increased plasma H_2CO_3. The decreased H_2CO_3 in the DCA-treated animals is the result of an effect of the drug; the increased H_2CO_3

TABLE 11

Chronic Effects of DCA upon Plasma and Tissue Acid-Base Balance in the Intact Rat[1,2]

	PLASMA			TISSUE			
					Intracellular values		
	pH	HCO_3^- mEq/L	H_2CO_3 mEq/L	Total CO_2 mEq/kg wet weight	HCO_3^{-2}	$H_2CO_3^2$	pH
CONTROLS	7.38	20.45	1.05	13.19	15.10	1.12	7.23
	7.24	16.47	1.19	12.52	14.53	1.27	7.16
	7.42	20.07	.96	13.52	15.42	1.03	7.27
	7.43	21.61	1.00	12.35	13.71	1.07	7.21
	7.39	20.34	.99	11.96	13.56	1.06	7.21
	7.33	20.56	1.20	11.88	12.89	1.28	7.10
	7.37±.028	19.92±.72	1.07±.042	12.57±.27	14.20±.39	1.14±.045	7.20±.025
DCA	7.52	23.29	.88	12.74	15.29	.94	7.28
	7.50	19.31	.77	11.65	13.99	.82	7.31
	7.49	22.07	.90	12.28	14.63	.96	7.25
	7.50	21.33	.85	12.98	15.94	.91	7.32
	7.48	24.17	1.00	12.73	15.08	1.07	7.22
	7.43	19.51	.91	13.23	16.39	.97	7.30
	7.49	21.72	.89	12.67	15.54	.95	7.29
	7.43	22.42	1.04	12.52	14.96	1.11	7.20
	7.44	24.75	1.13	15.09	18.22	1.20	7.25
	7.48±.011**	20.63±.58	.93±.035**	12.88±.31	15.56±.40*	.992±.039*	7.27±.013*

[1]All means ± standard error.

[2]Concentrations in mEq/kg cell water.

TABLE 12

Acute Effects of DCA Upon Plasma and Tissue Acid-Base Balance in the Eviscerated Rat[1]

	PLASMA			TISSUE			
	pH	HCO_3^- mEq/L	H_2CO_3 mEq/L	Total CO_2 mEq/kg wet weight	Intracellular values		
					HCO_3^{-2}	$H_2CO_3^{2}$	pH
Controls	7.18	17.34	1.44	12.60	15.00	1.54	7.09
	7.19	11.73	.95	12.50	16.21	1.02	7.30
	7.40	14.28	.72	13.46	17.59	.767	7.46
	7.19	14.44	1.17	12.08	14.95	1.25	7.18
	7.23	18.62	1.38	11.33	13.00	1.48	7.05
	7.24±.041	15.28±1.22	1.13±.13	12.38±.35	15.35±.76	1.21±.14	7.22±.077
DCA	7.39	14.06	.721	13.66	17.92	.771	7.47
	7.47	13.71	.584	13.47	17.84	.625	7.56
	7.37	14.43	.776	13.45	17.49	.830	7.42
	7.27	17.62	1.17	12.51	15.16	1.25	7.18
	7.38±.041*	14.96±.90	.813±13	13.27±.26	17.10±.66	.809±.13	7.41±.081
Controls	7.27	12.81	.851	12.17	15.68	.911	7.33
	7.28	15.05	.993	12.00	14.94	1.06	7.25
	7.19	17.04	1.38	13.96	17.19	1.48	7.17
	7.32	11.93	.701	11.33	14.70	.75	7.39
	7.27±.026	14.21±1.14	.981±.15	12.37±.57	15.63±.56	1.05±.16	7.21±.048
DCA	7.41	14.86	.728	12.70	16.36	.779	7.42
	7.41	15.02	.736	12.40	15.87	.787	7.41
	7.47	10.24	.450	11.64	15.70	.481	7.61
	7.43	11.26	.526	11.68	15.53	.563	7.54
	7.44	11.79	.392	11.23	14.96	.419	7.65
	7.43±.011***	12.63±.97	.566±.07*	11.93±.27	15.68±.23	.606±.08*	7.53±.049**
Controls	7.36	14.04	.812	17.18	23.14	.868	7.52
	7.29	12.84	.828	14.31	17.97	.886	7.41
	7.33	12.90	.758	15.67	21.08	.811	7.51
	7.42	13.32	.638	14.99	20.14	.683	7.57
	7.38	10.37	.858	14.33	19.28	.918	7.42
	7.37	13.49	.725	14.66	19.52	.776	7.50
	7.36±.018	12.83±.52	.770±.033	15.19±.45	20.19±.73	.824±.035	7.49±.025
DCA	7.51	14.46	.562	15.78	21.28	.601	7.65
	7.48	14.53	.605	13.68	18.03	.647	7.54
	7.48	12.26	.511	14.32	19.42	.547	7.65
	7.49	14.27	.582	15.80	21.30	.623	7.63
	7.43	12.54	.585	15.67	21.34	.626	7.63
	7.44	13.70	.624	14.61	19.52	.668	7.57
	7.47±.022***	13.63±.41	.578±.016***	14.98±.37	20.14±.56	.619±.017***	7.61±.018**

[1]All means ± standard error.

[2]Concentrations in mEq/kg cell water.

TABLE 12A

Analysis of Variance for Cell pH in Table 12

Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F	p
DCA treatment	1	.239	.239	19.45	.001
Between experiments	2	.304	.152	12.37	.001
Interaction	2	0.018	.009	1.37x	0.20
Within groups	24	.295	.01229		
Total	29	.873			

This table shows that DCA treatment will change cell pH significantly. However, the cell pH's obtained from day to day are significantly different. The lack of interaction shows that DCA will exert the effect upon each experimental day, beginning at any control level.

TABLE 13

Acute Acid-Base Effects of DCA

in Intact Animals[1]

	Plasma		Muscle
	pH	Total CO_2 mEq/L	Total CO_2 mEq/kg
Control	7.48	21.99	12.88
	7.43	19.76	10.97
	7.47	21.19	12.91
	7.49	19.65	13.16
	7.47	24.49	11.01
	7.47±.01	21.42±.89	12.18±.41
DCA-Treated	7.49	20.30	12.82
	7.46	22.16	13.15
	7.47	21.45	12.91
	7.47	22.26	12.02
	7.45	23.53	13.88
	7.47±.007	21.94±.53	12.96±.30

[1] All means ± standard error.

in the diet-induced potassium deficit is caused by a compensatory hypoventilation to correct the alkalosis. DCA-treated animals should also hypoventilate to compensate for the alkalosis, but the effect observed is that of a hyperventilation.

A direct effect of DCA to cause an extracellular alkalosis is shown by the data in Table 12 . No potassium losses occurred in these animals; nevertheless, an extracellular alkalosis was observed. These data confirm the recent observations of Grollman and Gamble (1959), who reported that animals treated with DCA exhibited an extracellular alkalosis even when potassium losses were prevented by low-sodium diets. They attributed this alkalosis to a direct effect of DCA. The data from DCA-treated animals shown here indicate that plasma H_2CO_3 decreases after treatment. Grollman and Gamble reported an increased H_2CO_3. This is rather surprising since both animal groups received DCA and neither group exhibited potassium losses. However, the animal groups were different in several respects. The eviscerated animals had undergone a traumatic operation and had been adrenalectomized as well. They also had developed a marked metabolic ~~alkal~~acidosis secondary to the evisceration. Grollman and Gamble's animals had been on low potassium-low sodium diets for several days and were in reasonably good health. However, no explanation of the difference in H_2CO_3 is apparent from any of these differences.

The most striking effect of DCA shown by these experiments is the intracellular alkalosis induced by DCA. Calculations of intracellular pH from CO_2 data show that intracellular pH is increased, both in the intact and the eviscerated animal. This alkalosis is not in agreement with other studies which show that DCA administration causes a cellular acidosis. However, other studies in which cellular pH has been measured, and not assumed on the basis of extracellular acid-base changes, have in-

cluded other variables, e. g., low potassium diets. These experiments may have measured the resultant of two opposing effects so that any direct effects of DCA could have been masked. Differences in the effects of DCA administration and potassium deprivation upon extracellular acid-base changes have been pointed out in the introduction to this section. The possible differences between DCA treatment and potassium depletion at the cellular level have not been discussed.

The possibility exists that sodium and hydrogen ions are both pumped from the cell to the extracellular fluid by the same extrusion mechanism. If DCA enhanced this process, a depletion of cellular hydrogen ions would occur, and the observed alkalosis would be explained. However, H_2CO_3 concentrations in the extracellular fluid are decreased, and HCO_3^- concentrations are increased. These findings are not compatible with the idea that hydrogen ions are moved from the cell to the extracellular fluid. Potassium deprivation, on the other hand, would cause the sodium-hydrogen pump to slow down, because a normal potassium extracellular concentration is necessary for efficient function of the pumping mechanisms. This would lead to cellular acidosis and sodium accumulation, both of which have been observed in potassium deficiency. Thus, potassium deficiency and DCA administration could cause different effects upon cellular pH.

Because the enhanced extrusion of hydrogen ion caused by DCA will not explain all of the observed effects, other possibilities should be explored. In many experiments the accumulation of CO_2 in the muscle of DCA-treated animals is quite marked, and extracellular pCO_2 is lowered. This could mean that the CO_2 produced in the cell is not in equilibrium with the extracellular fluid. Because equal tensions of CO_2 in all body compartments are assumed in the calculations of intracellular pH, a disequilibrium of this type would indicate a cellular alkalosis, since

a low cellular pCO_2 would be assumed. Equilibrium distortions of this type have been reported in brains of acetazolamide-treated animals (Koch and Woodbury, 1958). These authors argued that cell pH calculations based on CO_2 data were not reliable in their experiments because other evidence indicated that the cell was acidotic, rather than alkalotic as indicated by the CO_2 calculations. The same argument could be applied to muscle in the experiments reported here. However, there is no evidence that DCA can interfere with CO_2 equilibrium in this fashion. The final resolution of this problem will come when simultaneous determinations of cellular pH utilizing CO_2 data and another method not dependent upon CO_2 distribution are completed. Dimethyloxazolidine dione is a tool that will be useful in such studies.

The overall acid-base picture of the animals is confusing. Intact animals have normal acid-base pictures with the exception of a respiratory alkalosis induced by ether inhalation. The control eviscerated animals have a marked metabolic acidosis. Yet both groups of animals respond to DCA treatment by increasing extracellular HCO_3^- and decreasing extracellular pCO_2. This action of DCA does not seem to be affected by previously existing acid-base distortions.

In summary, DCA causes a cellular alkalosis associated with a confusing acid-base picture. The explanation of this alkalosis is not readily apparent , but it is real. The findings presented here will force a revision in thinking concerning the effects of steroids upon acid-base balance.

VI. EFFECTS OF DCA UPON SKELETAL MUSCLE LYSINE CONCENTRATION

A. Introduction

Lysine concentration changes in skeletal muscle have been reported in potassium deficiency experiments. Eckel, Pope, and Norris (1954, 1958) showed that DCA administration and/or low-potassium diets increased muscle concentrations of lysine. Iacobellis, Muntwyler, and Dodgen (1956) confirmed this observation. Vernadakis (1957) showed that lysine concentrations were lowered in cerebral cortex subsequent to DCA treatment.

It has been proposed that lysine enters muscle cells to ameliorate the cation deficit observed in the muscle of potassium-deficient animals. Eckel, Pope, and Norris (1958) compared changes in muscle lysine concentration with metallic cation deficits and concluded that lysine was a significant cation in potassium-depleted muscle. Further, they proposed that lysine, rather than hydrogen ion, was the mobile cation which made up the cation deficit observed when muscle potassium loss was greater than muscle sodium accumulation. Lysine feeding experiments substantiated the proposal that this amino acid could serve as a mobile cation (Eckel, Pope, and Norris, 1958). The observation that lysine entered muscle cells in lieu of hydrogen ions following a muscle potassium depletion suggested that the decrease in cellular pH thought to take place in such circumstances might not occur.

Lysine concentration changes in muscle could be of significance for other reasons. Amino acids have been connected with active transport, usually with the insinuation that the substance is related to, or

synonymous with the sodium carrier. Terner, Eggleston, and Krebs (1950) implicated glutamic acid in the transport of potassium in isolated brain slices and retinal sections. Eyring and Dougherty (1955) speculated that histamine is the carrier substance, at least in frog muscle. Vernadakis (1957) interpreted brain amino acid data as indicating that glutamine is a carrier for sodium transport across brain cells. Conway and Duggan (1958) demonstrated that amino acids affected sodium transport. They found that sodium transport out of yeast cells was increased by an increase in the concentration of lysine, and other basic amino acids, outside the cell. A possible basic amino acid-Na linkage in a transport system is indicated by these data.

Experiments necessary to produce information concerning lysine function in carrier processes were beyond the scope of this investigation. The lysine studies presented here were conducted primarily for the purpose of correlating lysine concentration changes in muscle with shifts of muscle potassium and hydrogen ion concentrations.

B. Methods

Amino acid experiments were performed on intact and eviscerated animals in the acute experiments outlined in IV B. No chronic amino acid data was collected during these studies. All sampling and analytical procedures are cited in Section III.

C. Results and Discussion

The results of the lysine studies are shown in Table 14. Lysine concentrations in skeletal muscle of intact and eviscerated rats are not altered by DCA. The data in Table 14 do not confirm the data of Eckel, Pope, and Harris (1958) which showed that lysine concentrations in muscle were increased by DCA treatment. However, the changes in muscle potassium

TABLE 14

Effect of DCA on Lysine Concentrations

in Skeletal Muscle

Intact		Eviscerated	
Control	DCA-treated	Control	DCA-treated
mg/100 g wet tissue			
78	70	90	100
74	70	115	120
73	85	96	110
80	75	119	105
80		115	66
		105	135
		80	120
			79
			55
			115
mean = 77	mean = 75	mean = 102.4	mean = 100.5
std. error = ± 1.48	std. error = ± 3.53	std. error = ± 5.55	std. error = ± 8.16
p = > .05		p = > .05	

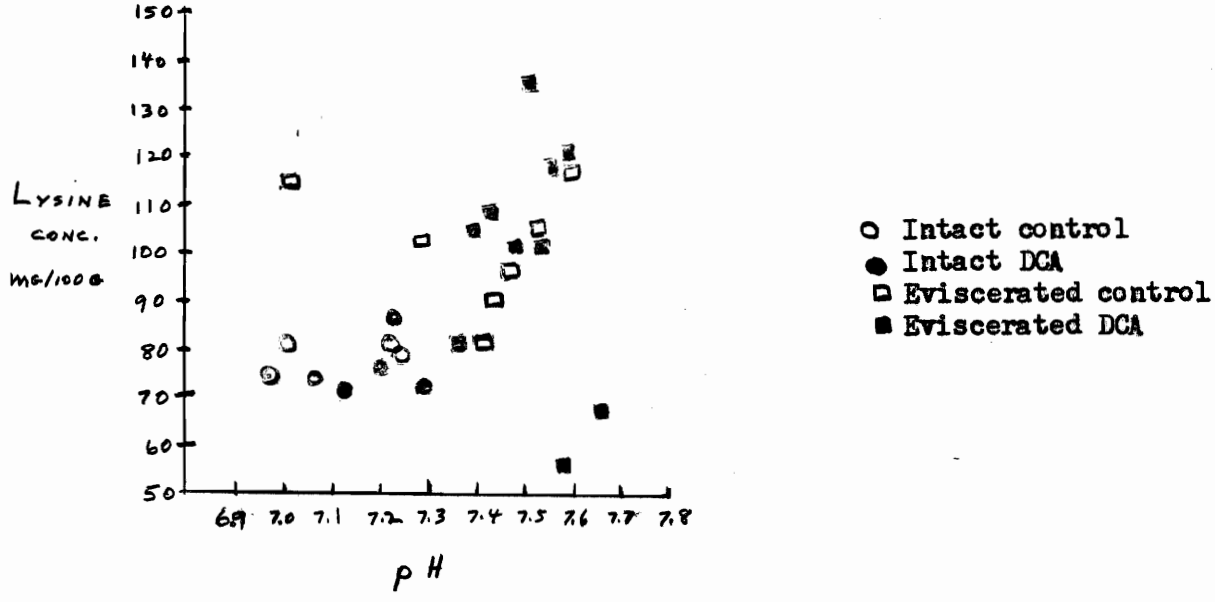
were not similar. In the experiments presented here, DCA treatment was continued for only 6 hours. No potassium was lost from muscle during this time (see Table 10). The experiment of Eckel, Pope, and Norris was a long-term study in which marked decreases in muscle potassium were observed. Therefore, it appears likely that lysine concentrations in muscle increase secondary to a muscle potassium loss caused by either a low-potassium diet and/or the administration of DCA. Further evidence to support the proposal has been presented by Eckel, Pope and Norris (1958). They fed rats a low-potassium, low-protein diet which did not deplete muscle potassium even after 21 days. No lysine accumulation was observed in these experiments. The muscle data shown in Table 14 do not confirm the effect of DCA upon cerebral cortex observed by Vernadakis (1957). She found that DCA administered for only 6 hours caused a significant decrease in brain lysine concentration.

No evidence has been obtained to show that lysine concentration changes are significant in muscle acid-base fluctuations. Table 15 shows a comparison of cell pH and muscle amino acid concentrations. The correlation coefficients calculated for each animal group show that the relation between cell pH and lysine concentrations is not predictable. The lysine data reported here do support the findings of Eckel, Pope, and Norris (1950) which showed that lysine concentrations increased when the muscle cell became more acidotic. The wide range of cell pH's, 7.05-7.65, shown here is much greater than the pH ranges reported by Eckel, Pope, and Norris yet these data indicate no lysine changes. Recently, evidence has been published which indicates that lysine accumulates in muscle even though the cell loses potassium and shows no pH changes (Eckel,

TABLE 15

Comparison of Muscle Lysine Concentration with Cell pH

Intact		Eviscerated	
Cell pH	Muscle Lysine Conc. mg/100 g	Cell pH	Muscle Lysine Conc. mg/100 g
6.97	74	7.05	115
7.01	80	7.18	86
7.07	73	7.30	115
7.11	70	7.41	80
7.20	75	7.41	115
7.22	80	7.42	79
7.23	78	7.42	110
7.23	85	7.46	96
7.27	70	7.47	100
		7.51	135
		7.52	105
		7.52	105
		7.54	80
		7.56	72
		7.57	120
		7.57	55
		7.63	123
		7.65	66
r = .153; p = > .05		r = -.201; p = > .05	



Botschner, and Wood, 1959). The present report agrees with the observation that lysine concentration changes are not related to muscle pH changes.

The intact animals have lower muscle lysine concentrations than those of the eviscerated animals, as shown in Table 14. No apparent explanation is forthcoming for this discrepancy.

VII. THE BARIUM-SOLUBLE FRACTION

A. Introduction

Conway and Fearon (1944) have presented data which show that a significant fraction of the total acid-labile CO_2 in skeletal muscle is not derived from HCO_3^- and H_2CO_3. They found that a portion of the CO_2 extracted from muscle did not precipitate when $BaCl_2$ was added to an alkaline muscle extract. The authors concluded that this barium-soluble fraction represented some bound form of CO_2 which was not available for cell buffering, but which yields CO_2 in acid solutions. Therefore, they reasoned that the use of acid-labile CO_2 measurements for the calculation of distributions of HCO_3^- and H^+ was not valid unless a correction was made for the CO_2 liberated from the barium-soluble fraction.

Hydrogen and HCO_3^- ion distributions were calculated by these authors from CO_2 data which had been corrected for the barium-soluble fraction. They found that extracellular-intracellular concentration ratios of both H^+ and HCO_3^- agreed with ratios predicted by the existence of a Donnan equilibrium between the two fluid compartments. This indicated that the muscle cell was permeable to bicarbonate, and that hydrogen ions were distributed passively across the muscle membrane. Both indications disagreed with current ideas concerning membrane permeability and hydrogen ion transport. Furthermore, if a barium-soluble fraction were actually present in muscle, the standard calculations of intracellular pH, which utilize CO_2 data, would be in serious error. Therefore, it was decided to repeat the experiment of Conway and Fearon, and to study more carefully the characteristics of this fraction if its existence were proven.

B. Methods

Two types of experiments were completed. Firstly, the presence or absence of a barium-soluble fraction was determined by repeating the original experiment of Conway and Fearon. A muscle sample was extracted in the cold with 0.2 KOH. The acid-labile CO_2 in an aliquot of the extract was determined by methods described in Appendix 1. The barium-soluble fraction was detected by adding $BaCl_2$ to an aliquot, separating the $BaCO_3$ precipitate by centrifugation, and analyzing the supernatant for CO_2. The presence of CO_2 in the supernatant after the addition of $BaCl_2$ and separation of the $BaCO_3$ was interpreted as indicating a barium-soluble fraction.

Secondly, ultracentrifuge experiments were carried out to study the nature of the barium-soluble fraction. The experiments were designed to test two hypotheses: 1, that the ordinary laboratory centrifuge was not powerful enough to separate the $BaCO_3$ from the thick KOH solution; and 2, that protein was serving as a suspension vehicle to prevent $BaCO_3$ precipitation (see Kirk, 1950, for a discussion of the suspending properties of muscle extracts). The first hypothesis was studied by adding the $BaCl_2$ to an aliquot of muscle extract before centrifugation at 100,000 X g for 2 hours. The second hypothesis was examined by adding $BaCl_2$ to a muscle extract after the extract had been centrifuged for 2 hours at 100,000 X g.

C. Results and Discussion

The data of the centrifugation experiments are shown in Tables 16 and 17. The values given in Table 16 indicate that a barium-soluble fraction is present under these experimental conditions. However, the size of this fraction compared to the total CO_2 content of the muscle is

TABLE 16

Daily Variations of the Barium Soluble Fraction

Experiment	Number of Determinations	Total CO_2, mm/kg	Barium Soluble Fraction mm/kg
1	4	8.46	5.31
2	4	9.32	3.08
3	10	9.17	0.57
4	8	11.57	2.12
5	8	11.08	trace
6	8	8.79	7.11
7	6	9.21	1.09
8	8	11.16	0.89
9	8	8.58	0.77

not as reproducible as the fractions reported by Conway and Fearon. Table 17 shows that the barium-soluble fraction observed in any particular experiment cannot be centrifuged out even after a 2-hour exposure to a force 100,000 X g. Table 17 further shows that any interfering substance which was extracted with the KOH could not be separated by centrifugation.

The experiments described above do not explain the nature of the barium-soluble fraction; however, they indicate that the barium-soluble fraction is probably not a suspension of crystalline $BaCO_3$. Conway and Fearon claimed to have eliminated the possibility that the carbamino compound was responsible for the appearance of the barium-soluble fraction. At present, this is all the direct knowledge we have about the existence of a barium-soluble fraction.

However, the fallacious nature of the barium-soluble fraction is indicated by experimental results reported by other workers. When the barium-soluble fraction was subtracted from the total acid-labile CO_2, Conway and Fearon calculated the pH of the muscle cell to be 6.0. Recent work with dimethyloxazolidine-diones (DMO) has shown that the pH of the skeletal muscle cell is approximately 7.0 (Waddell and Butler, 1959). DMO is a weak acid that distributes itself across cell membranes in the same manner as carbonic acid; that is, the membrane is permeable to the non-disassociated form but will not freely pass the ionized form of the acid. Waddell and Butler used the same assumptions for calculating cellular pH from DMO data as are commonly utilized for pH calculations with CO_2 data. The fact that results with DMO agree with those calculated from <u>total</u> acid-labile carbon dioxide, but do not agree with those calculations corrected by a barium-soluble fraction, indicates that the existence of a barium-soluble fraction is doubtful. Direct measurements

TABLE 17

Ultracentrifuge Effects on the Barium-Soluble Fraction of Muscle

	CONTROL		CENTRIFUGED*	
	Experiment 1†	Experiment 2	Experiment 1	Experiment 2
Total CO_2 in muscle	9.53	8.02	9.11	7.57
Barium-soluble fraction (barium added after extract centrifugation.)	5.67	4.00	5.67	3.57
Barium-soluble fraction (barium added before extract centrifugation.)	5.67	4.00	5.07	4.53

*40,000 r.p.m. for 2 hours.

†Each number is the average of three determinations. All values are in mM/kg wet weight of muscle.

with glass electrodes in crab muscle and squid axon confirm the muscle cell pH to be approximately 7.0 (Caldwell, 1958). The maintenance of a cell pH greater than 6.3, even in the face of very high extracellular hydrogen ion concentrations, has been demonstrated by metabolic experiments. Hill (1955) has shown that lactic acid production continues even though extracellular pH falls to a value of 5. Lactic acid production normally ceases at pH 6.3. If hydrogen ions were distributed according to the Donnan equilibrium, as predicted by the barium-soluble data of Conway and Fearon, the internal pH of skeletal muscle should be 1.5 pH units lower than external pH, as shown by the following calculation:

$$\text{Internal pH} - \text{External pH} = \frac{\text{Membrane potential}}{59}$$

For skeletal muscle, membrane potential = -90 mV. At an external pH of 5, the internal pH should be:

$$\text{Internal pH} = -1.52 + 5 = 3.48$$

Thus, the conclusions of Hill argue strongly against the presence of a barium-soluble fraction in muscle.

Little work has been done on the problem of the barium-soluble fraction since Conway and Fearon first made their report in 1944. However, enough information is available to indicate strongly that the barium-soluble fraction is an artifact. Although muscle acid-base physiology is not being retarded because of the barium-soluble fraction, the elucidation of its true nature poses an interesting problem.

SUMMARY

It has been found that the primary effects of DCA upon skeletal muscle, particularly the effect upon sodium movements, are masked by electrolyte shifts that are secondary to muscle potassium depletion. The muscle potassium depletion is caused by an action of DCA to promote potassium excretion via the kidney and digestive tract. When muscle potassium losses were prevented in an eviscerated preparation, a direct effect of DCA to decrease cellular sodium was demonstrated in this tissue for the first time. In addition, direct actions of this steroid to increase total muscle carbon dioxide and to cause an extracellular alkalosis were observed in experiments where no potassium losses occurred. Calculations of intracellular pH based on data derived from carbon dioxide measurements reveal that control animals had a muscle pH in the vicinity of 7. DCA treatment of both intact and eviscerated animals caused the muscle cell to become alkalotic. These experiments indicated that DCA exerts an effect upon muscle acid-base balance in the absence of any changes in muscle potassium.

Amino acid studies failed to support the observations of other workers that basic amino acids, particularly lysine, are mobile cations in muscle acid-base changes. No change in the concentrations of basic amino acids in muscle cells sufficient to counteract metallic cation deficiencies was detected. No reproducible changes in basic amino acids were observed following acid-base changes in the muscle.

Investigations of the barium-soluble fraction of muscle showed that the fraction is highly variable in size, and that it is not a protein suspension of barium carbonate.

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APPENDIX I

A Simple Method for the Analysis of Total Carbon Dioxide in Tissue and Plasma*

Theory. Carbon dioxide (CO_2), derived either from carbonic acid or bicarbonate ions, is released when a tissue sample is placed in a solution of ferric fluoride (FeF_3). The FeF_3 solution acts as an acid which can expel CO_2 and also inhibit enzymatic production (Danielson and Hastings, 1939) of additional carbon dioxide.

The amount of CO_2 released can be measured by absorbing the gas in a solution of barium hydroxide ($Ba(OH)_2$). The CO_2 is hydrated to form H_2CO_3 which reacts quantitatively with the barium ion to form an equivalent amount of insoluble barium carbonate, as shown in the equation below:

$$CO_2 + H_2O + Ba(OH)_2 \longrightarrow BaCO_3 + 2H_2O.$$

The decrease in $Ba(OH)_2$ concentration caused by the formation of insoluble $BaCO_3$ is directly proportional to the amount of CO_2 absorbed. One mole of CO_2 forms one mole of $BaCO_3$, and each mole of $BaCO_3$ formed causes the disappearance of one mole of $Ba(OH)_2$. Thus, if the amount of $Ba(OH)_2$ present before and after CO_2 absorption occurs is measured, the difference in $Ba(OH)_2$ concentration is directly proportional to the amount of CO_2 absorbed. The amount of CO_2 absorbed is, in turn, equivalent to the CO_2 present in the tissue.

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*Specific suggestions of Dr. R. E. Eckel, Department of Medicine, Western Reserve University, Cleveland, Ohio, were utilized in the development of this method. Some details of this method have recently been published (Eckel, Botschner, and Wood, 1959).

The decrease in $Ba(OH)_2$ concentration, and consequently an estimate of tissue CO_2, can be measured by a simple titration with a strong acid. The $BaCO_3$ will not react with strong acid until all the excess $Ba(OH)_2$ is neutralized. However, $BaCO_3$ will dissolve in acid if the titration is continued beyond the neutralization point of the hydroxyl ions of the $Ba(OH)_2$. Therefore, the titration must be stopped before the $BaCO_3$ reacts, but after the hydroxyl ions have all been neutralized, if the amount of HCl added is to be directly proportional to the amount of CO_2 originally present in the tissue. This is accomplished by choosing an indicator that changes color when the hydroxyl ions are neutralized.

Phenolphthalin would have been a suitable indicator for this hydroxyl ion neutralization point if the color change from red to colorless at pH 8 could have been easily detected. Unfortunately, this was not the case, so another detection method for the end-point was devised. Phenolphthalin was combined with a mixture of methyl red and bromcresol green to give a wine-red color. At pH 8 this mixture changes color from wine-red to forest-green; the green color is the color of the indicator mixture at any pH greater than 5.1. The color change stops the titration at a pH slightly below 8. The high pH prevents the reaction of $BaCO_3$. Reference to a standard titration curve for a strong acid and a strong base (Kolthof and Sandell, 1945) shows that 99.99% of the hydroxyl ions have been titrated at this end point.

Reagents. a. Hydrochloric acid, 0.200 M.

b. Barium hydroxide, saturated solution.

c. Mixed Indicator Stock Solution. This is prepared by adding

2 ml of saturated $Ba(OH)_2$ to 500 ml H_2O, in addition to 3 ml of brom-cresol green (0.1% in 95% ethyl alcohol) and 2 ml of methyl red (0.1% in 95% ethyl alcohol). The solution is then diluted to one liter with distilled water. The final solution should be a dark forest-green color.

d. Absorption Solution. 7.5 ml of saturated $Ba(OH)_2$ is diluted to 100 ml with the mixed indicator stock solution. Saturated phenol-phthalein (in 95% ethyl alcohol) is dropped slowly into the absorp-tion solution until a permanent wine-red color is observed.

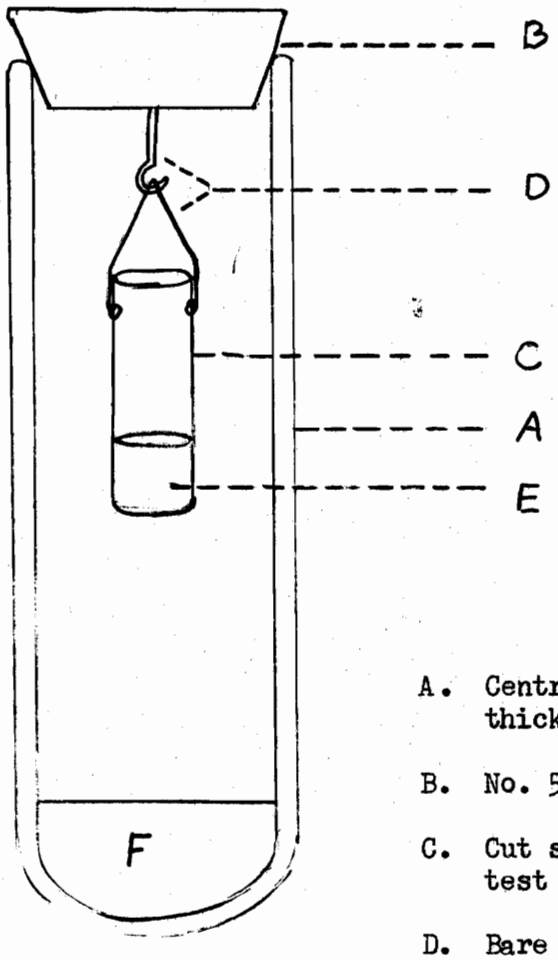
e. Ferric Fluoride. Equal volumes of a 10% aqueous solution of ferric sulfate and a 4% aqueous solution of sodium fluoride are mixed slowly. This solution should be light brown color, reasonably clear, and have a pH of approximately 4.5.

Procedure. The details of a tissue total carbon dioxide determina-tion are as follows:

1. Absorption tubes are fashioned from the appropriate parts as shown in figure 2.

2. Two ml of absorption solution are pipetted into the absorption bucket, and 3 ml of ferric fluoride solution are pipetted into the centrifuge tube. The test tube bucket is suspended on the hook in the stopper, as shown in figure 1, and stopper is then forced tightly into place. The entire assembly is weighed to the closest milligram.

3. Stopper B with its attached bucket is placed in a centrifuge tube containing some saturated $Ba(OH)_2$ in order to protect it from atmospheric CO_2 during the sampling procedure. A 0.5-1.5 g tissue is rapidly placed in the centrifuge tube containing ferric fluoride. The sample tube is sealed with its original stopper and weighed again.



A. Centrifuge tube, 25 X 120 mm, thick-walled.

B. No. 5 rubber stopper.

C. Cut section of ordinary pyrex test tube, 12 X 50 mm.

D. Bare copper wire.

E. Absorption solution.

F. Ferric fluoride.

Fig. 3. Carbon Dioxide Absorption Tube

Sample weight is equal to the difference between the weight of the tube in 2. and 3. Controls are obtained by exposing blank tubes to the atmosphere for the same length of time that the sample tubes were open.

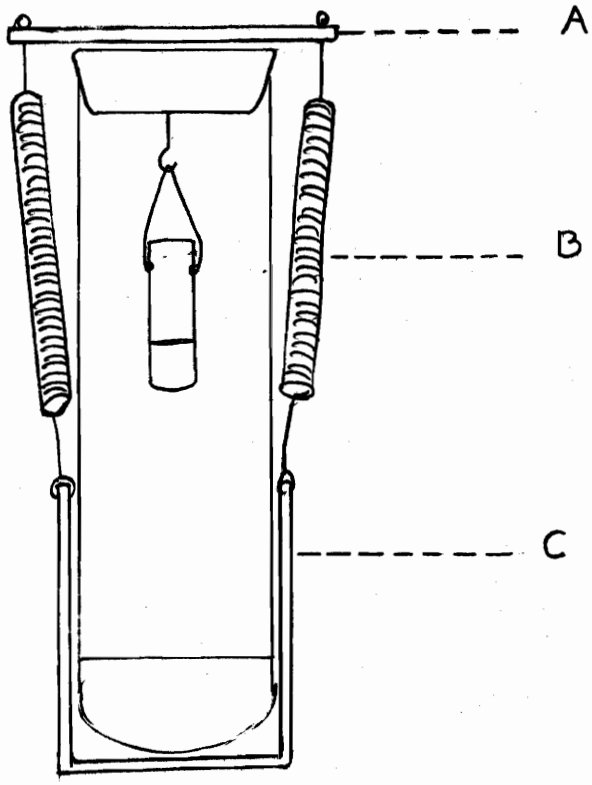
4. The gas-tight absorption tube from 3. is placed in a tension clamp as shown in figure 3. The clamp assembly is placed in a water bath at a temperature of 50° C and shaken vigorously for 4 hours.

5. At the end of a 4 hour period of shaking, the samples are cooled and placed in the titration assembly shown in figure 3. The stream of CO_2-free air passing through the needle B prevents the absorption of atmospheric CO_2 by maintaining a CO_2-free atmosphere in the bucket.

6. The wine-red absorption solution, containing a white precipitate of barium carbonate, is titrated to a forest-green endpoint with 0.2000 M HCl.

7. Plasma total CO_2 is determined by substituting a pipetted aliquot of plasma for a tissue sample mentioned above in section 3. Optimum sample size is 1.0 ml, but 0.5 ml samples are easily analyzed. Smaller samples, e.g., 0.2 ml, are measurable, but precision is drastically reduced.

Calculations. The decrease of $Ba(OH)_2$ concentration as a result of CO_2 absorption is directly proportional to the amount of CO_2 in the tissue sample, as shown above. The amount of HCl needed to titrate the solution after CO_2 absorption is inversely proportional to the amount of CO_2 released from the unknown. In other words, a blank or control tube would require a maximum amount of HCl to neutralize the $Ba(OH)_2$, while a tube which had absorbed a large quantity of CO_2 would require a much smaller amount to reach the same endpoint.



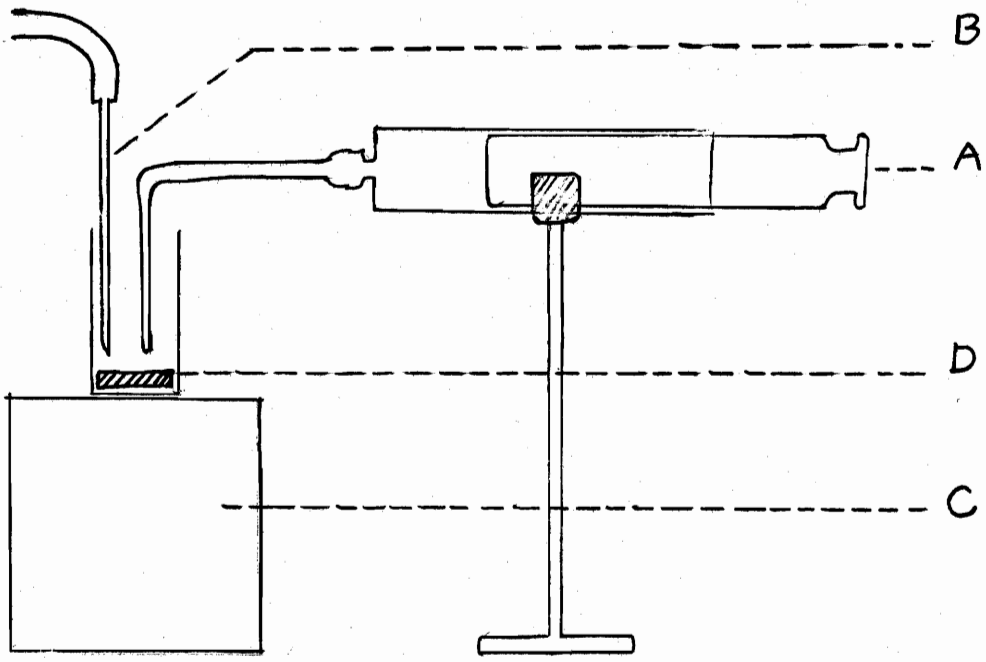
A. Metal bar, 2 3/8" X 1"

B. Heavy spring

C. Brass cylinder, 1 3/8" in diameter X 1 1/2" high

Fig. 4. Tension Clamp* Containing Absorption Tube

*Fabricated by Aldo Gabardi



A. Syringe microburet (Lazarow, 1950).

B. No. 18 needle connected to compressed air source. This air is made CO_2-free by passing it through absorption tubes containing Ascarite and Barylyme.

C. Magnetic stirrer.

D. Glass-enclosed stirring bar

Fig. 5. Titration Assembly

The amount of CO_2 in a tissue sample is calculated from the following formula:

$$\text{muscle sample total } CO_2 \text{ in mM/kg wet weight} = \frac{(\text{ml control} - \text{ml sample})(\text{HCl titer})}{\text{weight sample in grams}}$$

The HCl titer is derived from the equation:

$$Ba(OH)_2 + 2HCl \rightleftarrows BaCl_2 + 2HOH$$

One mole of $Ba(OH)_2$ is the equivalent of one mole of $BaCO_3$, or one mole of tissue CO_2. Therefore, one mole of HCl is equivalent to one-half mole of tissue CO_2. For example, the HCl used in a typical titration was 0.1983 normal. In terms of carbon dioxide equivalents, one ml of HCl is equal to $\frac{198.3}{2}$ = 99.2 uM of CO_2. This figure, 99.2, would be the HCl titer referred to in the equation above <u>for this particular normality</u> of HCl. This titer may be derived by a direct, separate standardization of the HCl against standard base, or by including standards in the tissue analysis from which the acid titer can be calculated. Experience has shown that either method is satisfactory.

Plasma samples are calculated by multiplying the total amount of CO_2 found in the plasma sample by a dilution factor.

<u>Errors</u>. The accuracy and sensitivity of this method for determining total CO_2 in tissue are not limited by the chemical reactions employed in the determination, or by lack of precision in the measuring devices. The endpoint is extremely sharp, and the syringe microburette is an extremely precise burette and/or pipette. These two factors were studied by pipetting samples of the absorption solution with the syringe microburette, and titrating the samples with 0.2 M HCl. The amount of absorption solution selected required the addition of approximately 360 µL of HCl to reach an endpoint. Near the endpoint, the addition of only

1.5 μL of HCl produced a sharp color change. For this size sample, repeated titrations agreed with each other within ± 1%, or lower, of the mean value of all the volumes of HCl added.

The accuracy and sensitivity of the method under the actual conditions of sample analysis were studied by analyzing known amounts of a standard solution of Na_2CO_3. A volume of the standard solution was pipetted into the sample tube, and the analysis was completed according to the directions above. The total amount of carbonate added was in the range of the total amount of CO_2 contained in a tissue sample of 1 g. The results of these studies are given in Table 18 . Under operational conditions, the overall accuracy is approximately ± 2% of the mean value of the determinations.

Finally, the accuracy of the method was studied by analyzing paired muscle samples. In some experiments the samples were obtained by cutting the same muscle into two pieces; in other experiments, two different muscles were sampled. The results of these paired muscle experiments are shown in Table 19 . Most paired samples agree within 5-10%, but some pairs are in better agreement. This value compares favorably with the 10-20% agreement found when paired muscle samples are analyzed by the manometric method of Danielson and Hastings (1939).

Summary. The method is easily applied to the routine analysis of tissue samples. Only 5 minutes are required for the analysis of each sample, as compared to the 20-30 minutes required for manometric method. Also, the method is very simple so that few samples are ruined by mistakes in procedure. The manometric method is not reliable in this respect since one mistake in a long series of confusing manipulations will ruin a sample. The application of the chemical method for CO_2 de-

TABLE 18

Recovery of Known Amounts of Sodium Carbonate

by Chemical CO_2 Method

	No. Determinations	Concentration added (μM)	Concentration found (μM)*	Range
Standard No. 1	22	12.52	12.27 ± .023	12.05 - 12.50
Standard No. 2	18	15.61	15.38 ± .019	15.11 - 15.58
Standard No. 3	61	16.02	16.14 ± .017	15.90 - 16.33

*All values ± standard error.

TABLE 19

Comparison of Total Carbon Dioxide Concentrations of Paired Muscle Samples[1]

Experiment Number									
1		2		3		4		5	
Right Leg	Left Leg	Sample # 1 (left leg)	Sample # 2 (left leg)	Sample # 1 (left leg)	Sample # 2 (left leg)	Right Leg	Left Leg	Right Leg	Left Leg
12.01	12.82	12.35	12.52	14.69	14.03	12.08	11.33	12.17	13.07
12.40	11.83	13.52	13.19	16.03	15.32	12.51	12.36	13.51	12.70
10.44	10.69	11.96	11.88	14.95	14.67	12.88	13.16	12.42	12.86
11.99	12.11	12.28	12.74	13.87	15.10	12.82	11.90	12.00	12.57
12.58	13.06	12.73	13.23	14.49	14.09	14.87	14.15	11.79	11.64
		12.52	12.67	14.36	14.81	14.02	14.88	11.23	11.50
				14.73	14.00	12.60	12.42	13.96	14.13
				14.40	14.40			11.68	13.02
								11.33	12.21
								10.40	11.70

Average per cent difference between samples

Experiment Number	%
1	3.7
2	2.2
3	3.9
4	4.4
5	5.8

Average for all experiments 4.0

[1]All values mM/kg wet weight tissue.

terminations in this laboratory for several hundred CO_2 analyses has been highly successful.

Appendix 2.

General Form for Calculating Intracellular Electrolytes

P = Plasma concentration (mEq/L)

I = Interstitial fluid concentration (mEq/L)

T = Tissue concentration (mEq/Kg)

C = Intracellular concentration (mEq/L)

TI = Interstitial fluid of tissue concentration (mEq/Kg)

$I_{Na} = P_{Na} \times 1 / \%$ plasma $H_2O \times 0.95$

$I_K = P_K \times 1 / \%$ plasma $H_2O \times 0.95$

$I_{Cl} = P_{Cl} \times 1 / \%$ plasma $H_2O \times 1.05$

$I_{HCO_3} = P_{HCO_3} \times 1 / \%$ plasma $H_2O \times 1.05$

$I_{H_2CO_3} = P_{H_2CO_3} \times 0.98$

$I_{CO_2} = I_{H_2CO_3} + I_{HCO_3}$

$TI_{H_2O} = T_{Cl} / I_{Cl}$ (this value is now equivalent to L/kg tissue)

$TI_{Na} = TI_{H_2O} \times I_{Na}$

$TI_K = TI_{H_2O} \times I_K$

$TI_{CO_2} = TI_{H_2O} \times I_{CO_2}$

$C_{H_2O} = T_{H_2O} - TI_{H_2O}$ (T_{H_2O} is L/Kg or $\frac{\%\ \text{tissue}\ H_2O \times 10}{1000}$)

$$C_{Na} = \frac{T_{Na} - TI_{Na}}{C_{H_2O}}$$

$$C_K = \frac{T_K - TI_K}{C_{H_2O}}$$

$$C_{CO_2} = \frac{T_{CO_2} - TI_{CO_2}}{C_{H_2O}}$$

$$C_{H_2CO_3} = P_{H_2CO_3} \times 1.07$$

$$C_{HCO_3} = C_{CO_2} - C_{H_2CO_3}$$

$$C_{pH} = 6.1 \log \frac{C_{HCO_3}}{C_{H_2CO_3}}$$

THE DIRECT EFFECTS OF DESOXYCORTICOSTERONE ON SKELETAL MUSCLE ELECTROLYTE METABOLISM

by

Clarence Dean Withrow

An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

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The primary objectives of this research have been to study the direct effect of desoxycorticosterone acetate (DCA) upon skeletal muscle electrolyte and amino acid concentrations and to study the effects of this steroid upon muscle acid-base balance.

It has been found that the primary effects of DCA upon skeletal muscle, particularly the effects upon sodium movements, are masked by electrolyte shifts that are secondary to muscle potassium depletion. The muscle potassium depletion is caused by an action of DCA to promote potassium excretion via the kidney and digestive tract. When muscle potassium losses were prevented in an eviscerated preparation, a direct effect of DCA to decrease cellular sodium was demonstrated in this tissue for the first time. In addition, direct actions of this steroid to increase total muscle carbon dioxide and to cause an extracellular alkalosis were observed in experiments where no potassium losses occurred. Calculations of intracellular pH based on data derived from carbon dioxide measurements reveal that control animals had a muscle pH in the vicinity of 7. DCA treatment of both intact and eviscerated animals caused the muscle cell to become alkalotic. These experiments indicated that DCA exerts an effect upon muscle acid-base balance in the absence of any changes in muscle potassium.

In connection with the acid-base studies mentioned above, two subprojects were completed. Firstly, a chemical method for the determination of total tissue carbon dioxide was developed and applied routinely for tissue analyses. Secondly, the possibility that some of the acid-labile carbon dioxide in muscle might actually exist in a bound form, as advocated by Conway, was investigated. The results of these experiments showed that some carbon dioxide is present in a basic muscle extract

which is not precipitable with barium chloride. However, the magnitude, and in many experiments even the presence, of this "barium-soluble" fraction was much more variable than indicated by the data of Conway. Attempts to determine the nature of this fraction were generally unsuccessful. However, the possibility that extracted protein might be acting as a suspension agent was eliminated by a series of ultracentrifuge experiments.

Amino acid studies failed to support the observations of other workers that basic amino acids, particularly lysine, are mobile cations in muscle acid-base changes. No change in the concentrations of basic amino acids in muscle cells sufficient to counteract metallic cation deficiencies was detected. No reproducible changes in basic amino acids were observed following acid-base changes in the muscle.